UNIVERSITY
OF TASMANIA

Phenotypic and genotypic characterisation of altered penicillin-binding protein 3 (PBP3) mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*.

Elizabeth A. Witherden

BBiomedSc (Hons)

Submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

University of Tasmania

April 2014

Declaration of Originality

This thesis entitled "Phenotypic and genotypic characterisation of altered penicillin-binding protein 3 (PBP3) mediated resistance in *Haemophilus influenza*e and *Haemophilus haemolyticus*", describes original research conducted by the candidate within the School of Health Sciences (formerly School of Human Life Sciences) at the University of Tasmania, and contains no material which has been accepted for a degree or diploma by the University or any other institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material has previously been published or written by another person except where due acknowledgement is made in the text of the thesis, nor does the thesis contain any material that infringes copyright.

Candidate

Elizabeth A. Witherden

Date

4th April 2014

Statement of Authority of Access

The publishers of the manuscripts comprising Chapters 3 to 6 hold the copyright for that content, and access to the material should be sought from the respective journals. The remaining non-published content of the thesis may be made available for loan and limited copying in accordance with the Copyright Act 1968.

Candidate

Elizabeth A. Witherden

Date

4th April 2014

Statement of Co-Authorship of Jointly Published Work

Manuscript 1:

I was the primary author and lead investigator of manuscript 1, which comprises Chapter 3 of this thesis and was published as:

Witherden EA, Kunde D, Tristram SG (2012). An evaluation of SNP-based PCR methods for the detection of β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *J Infect Chemother*; 18 (4): 451-455.

Manuscript 2:

I was the primary author and lead investigator of manuscript 2, which comprises Chapter 4 of this thesis and was published as:

Witherden EA, and Tristram SG (2013). Prevalence and mechanisms of β-lactam resistance in *Haemophilus haemolyticus*. *J Antimicrob Chemother*; 68 (5): 1049-1053.

Manuscript 3:

I was the primary author and lead investigator of manuscript 3, which comprises Chapter 5 of this thesis and was published as:

Witherden EA, Kunde D, Tristram SG (2013). PCR screening for the N526K substitution in isolates of *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother*; 68 (10): 2255-2258.

Manuscript 4:

I was the primary author and lead investigator of manuscript 4, which comprises Chapter 6 of this thesis and has been accepted for publication as:

Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A (2014). The role of inter-species recombination of the *ftsI* gene on the dissemination of altered penicillin-binding protein 3 mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother*; In Press [doi: 10.1093/jac/dku022].

Manuscript 5:

I was the primary author and lead investigator with respect to study design, data analysis and manuscript write up of manuscript 5, which comprises Chapter 7 of this thesis. However, I wish to duly acknowledge Annette Sondergaard (Department of Clinical Medicine, Aarhus University, Denmark), for conducting the transformation experiments that generated the raw experimental data that significantly contributes to this chapter.

Candidate	Deputy Head of School
Elizabeth A. Witherden	**Dominic Geraghty**
Date	Date
4th April 2014	4th April 2014

Statement of Candidature Contribution to Thesis

The thesis comprises interlinked research investigations where the candidate **Elizabeth Witherden** is the lead investigator, however, the following people and institutions also contributed to the published and non-published work contained within the thesis as follows:

- Elizabeth Witherden (School of Health Sciences, University of Tasmania): Lead investigator responsible for design of each individual research project, laboratory and experimental analysis, data collection, data analysis, data interpretation, and is the lead author on all resultant manuscripts.

- Dr. Stephen Tristram (School of Health Sciences, University of Tasmania): Assisted with research project design, experimental techniques, and manuscript revisions.

- **Dr. Dale Kunde** (School of Health Sciences, University of Tasmania): Technical assistance in experimental work performed within the molecular biology laboratory, and manuscript revisions (Chapters 3 and 5).

- **Dr. Murray Adams** (School of Health Sciences, University of Tasmania): Thesis revisions.

- Dr. Paula Bajanca-Lavado (National Institute of Health, Department of Infectious Diseases, Portugal): Technical expertise and manuscript revision (Chapter 6).

- Dr. Alexandra Nunes (National Institute of Health, Department of Infectious Diseases, Portugal): Technical expertise in bioinformatics and data analysis (Chapter 6) as well as data interpretation and manuscript revision (Chapter 6).

- Annette Sondergaard (Department of Clinical Medicine, Aarhus University, Denmark): Conducted the transformation experiments that generated the raw data for Chapter 7.

- Bowen Zhang (School of Health Sciences, University of Tasmania): Assisted with the 16S rRNA data collection (Chapter 5).

Specific contributions to Published Chapters:

- Chapter 3; Elizabeth Witherden (75%), Stephen Tristram (20%), Dale Kunde (5%).

- Chapter 4; Elizabeth Witherden (80%), Stephen Tristram (15%), Bowen Zhang (5%).

- Chapter 5; Elizabeth Witherden (75%), Stephen Tristram (20%), Dale Kunde (5%).

- Chapter 6; Elizabeth Witherden (70%), Alexandra Nunes (15%), Stephen Tristram (10%), Paula Bajanca-Lavado (5%).

- Chapter 7; Elizabeth Witherden (70%), Stephen Tristram (10%), Annette Sondergaard (20%).

We, the undersigned agree with the above stated, "proportion of work undertaken" for each of the published or non-published chapters, which contribute to this thesis:

Candidate	Deputy Head of School
Elizabeth A. Witherden	**Dominic Geraghty**
Date	Date
4th April 2014	4th April 2014

Publications and Presentations at Conferences During PhD Candidature

A. Published Manuscripts:

Witherden EA, Kunde D, Tristram SG (2012). An evaluation of SNP-based PCR methods for the detection of β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *J Infect Chemother*; 18 (4): 451-5. [doi: 10.1007/s10156-011-0356-5].

Witherden EA, and Tristram SG (2013). Prevalence and mechanisms of β-lactam resistance in *Haemophilus haemolyticus*. *J Antimicrob Chemother*; 68 (5): 1049-1053. [doi: 10.1093/jac/dks532].

Witherden EA, Kunde D, Tristram SG (2013). PCR screening for the N526K substitution in isolates of *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother*; 68 (10): 2255-2258. [doi: 10.1093/jac/dkt189].

Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A (2014). The role of inter-species recombination of the *ftsI* gene on the dissemination of altered penicillin-binding protein 3 mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrobial Chemother*; In Press. [doi: 10.1093/jac/dku022].

B. Conference Presentations:

Witherden EA and Tristram SG (2012). PCR screening for β-lactamase-negative ampicillin-resistant strains of *Haemophilus influenzae*. Proceedings from the 22nd European Congress of Clinical Microbiology and Infectious Diseases Annual Meeting, 31st March – 3rd April, ExCel Exhibition and Convention Centre, London, United Kingdom, Poster p673.

Witherden EA and Tristram SG (2012). *Haemophilus haemolyticus* as a potential reservoir for *ftsI* gene mutations and altered penicillin-binding protein 3 (PBP3) mediated resistance in *Haemophilus influenzae*. Proceedings from the 22nd European Congress of Clinical Microbiology and Infectious Diseases Annual Meeting, 31st March – 3rd April, ExCel Exhibition and Convention Centre, London, United Kingdom, Poster p1301.

Witherden EA and Tristram SG (2012). A comparison of MIC based screening tests for β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. Proceedings from the 22nd European Congress of Clinical Microbiology and Infectious Diseases Annual Meeting, 31st March – 3rd April, ExCel Exhibition and Convention Centre, London, United Kingdom, Poster p672.

Witherden EA (2013). Molecular characterization of altered penicillin-binding protein 3 (PBP3) mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. Oral presentation proceedings from the Australian Society for Medical Research, Medical Research Week Postgraduate Student Awards, May 30 – June 7th University of Tasmania, Clinical School, Hobart, Tasmania.

Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A (2014). The role of inter-species recombination of the *ftsI* gene on the dissemination of altered penicillin-binding protein 3 mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. Proceedings from the Australian Society for Antimicrobials Annual Meeting, 20th-22nd February, Melbourne Exhibition Centre, Victoria, Australia, Poster p22.

Acknowledgements

Firstly, I wish to express my sincere gratitude and appreciation to all those who supported and helped me throughout the duration of my candidature. I would like to extend a deep and heartfelt thank you to Dr. Stephen Tristram and Dr. Murray Adams, my PhD supervisors, not only for their enthusiasm in my research area, but also for their invaluable guidance, knowledge and patience. Furthermore, I wish to extend my appreciation to Professor Dominic Geraghty, my Higher Degree Research coordinator, as well as Professor Steve Campbell Head of the School of Health Sciences (formerly the School of Human Life Sciences) and also Professor Madeleine Ball the former Head of School, for giving me the opportunity to study within the Faculty.

I would like to extend my gratitude to Dr. Dale Kunde from the University of Tasmania, for his technical expertise in molecular PCR assays, and would also like to acknowledge Dr. Lea-Ann Kirkham from the School of Paediatrics and Child Health from the University of Western Australia, Perth, for provision of some of the XV-dependent *Haemophilus* isolates characterised in the studies, comprising Chapters 4-7.

I sincerely thank all additional collaborators, especially Dr. Paula Bajanca-Lavado and Dr. Alexandra Nunes from the Department of Infectious Diseases from the National Institute for Infectious Disease Dr. Ricardo Jorge, Lisbon, Portugal, for hosting and teaching me the pulse-field gel electrophoresis method and bioinformatics techniques used in the analysis of mosaic gene structures that

comprises Chapters 6 and 7 of this thesis, respectively. I would also like to ackowldege and thank Annette Sondergaard from the Department of Clinical Medicine, Aarhua University, Denmark, for her assistance in the collection of raw experimental data for Chapter 7.

I wish to also thank and acknowledge my fellow PhD candidates and the technical staff within the School of Health Sciences, for their general support, and company in both the molecular and microbiology laboratories.

I would like to acknowledge the generous support of the Clifford Craig Medical Research Trust (CCMRT) for their funding of the research projects presented in this thesis (T0018852-89 CCMRT), as without their generous support this thesis would never have been possible.

Finally, I would like to thank my family, and in particular my partner Guy, not only for their patience, but also for their general interest in my thesis. You have kept me grounded, motivated and sane throughout my candidature, and for that I am truly grateful.

Thank you

Yours Sincerely,

Elizabeth A. Witherden

General Abstract

Haemophilus influenzae is a significant opportunistic pathogen that causes a range of respiratory infections, including community-acquired pneumonia (CAP), acute exacerbations of chronic obstructive pulmonary disease (COPD) and acute otitis media (AOM). These infections frequently require antibiotic therapy for management, with antibiotics of the β-lactam class such as amoxicillin, cefaclor, and amoxicillin-clavulanate historically used as first line therapies. However, the efficacy of these antibiotics is currently threatened by the increasing prevalence of β-lactam resistance mediated by specific mutations in the *ftsI* gene that produce an N526K substitution in the encoded penicillin-binding protein 3 (PBP3), a protein that is the target of these antibiotics. This type of resistance, termed β-lactamase-negative ampicillin-resistance (BLNAR), is difficult to detect in the diagnostic laboratory, as many BLNAR isolates do not actually show an ampicillin resistant phenotype using standard susceptibility testing methods. As a result genotypic testing methods are being increasingly adopted for BLNAR detection. Furthermore, the recent recognition of *Haemophilus haemolyticus,* a close non-pathogenic relative of *H. influenzae*, in diagnostic specimens from the respiratory tract, has compounded the issue. This is because *H. haemolyticus* isolates are frequently mis-identified as *H. influenzae*, which further complicates the role of the diagnostic laboratory in guiding antibiotic therapy for infections involving *H. influenzae*.

A working strain collection comprising a total of 393 *Haemophilus* isolates was established and used for all the subsequent studies conducted in this thesis. Isolates were taken from; 1) the University of Tasmania (UTAS) culture collection (n=44),

that had been previously collected from cases of respiratory illness, and 2) the Genetics of Recurrent Otitis Media and Immunology in Toddlers (GROMIT) Study conducted in Perth, Western Australia, where 349 XV-dependent *Haemophilus* isolates were cultured from paediatric patients suffering AOM with healthy aged-matched controls.

In Chapter 3, the SNP-based PCR algorithm of Hasegawa et al. (2003) that has been widely used to detect BLNAR mediated resistance was evaluated against the less commonly used PCR algorithm of Nakamura et al. (2009). Although, the primer set (PBP3-S) of Hasegawa et al. (2003), that is designed to amplify susceptible (N526) isolates was 100% sensitive and specific for detecting N526K-positive BLNAR isolates by non-amplification, the primer set (PBP3-BLN) designed to amplify N526K isolates was unreliable (sensitivity 84%; specificity 26%). Similar findings were reported for the sensitivity and specificity of the Nakamura et al. (2009) primers for detecting the N526K substitution in BLNAR isolates by amplification (sensitivity 96%; specificity 26%). The poor performance of these PCR primers was attributed to the fact that the N526K substitution can be encoded by an AAT-AAG codon change at base pair (bp) position 1576-1578 of *ftsI,* as well as the recently described AAT-AAA codon change. As the PCR primers investigated were designed for detection of the N526K substitution encoded by the AAG SNP only, they failed to detect N526K encoded by AAA. A search of *ftsI* gene sequences available on GenBank revealed that the AAT to AAG or AAA codon changes occurred with equal prevalence in N526K-positive BLNAR isolates, suggesting that the prevalence of BLNAR isolates would go under-reported when these PCR algorithms were used for BLNAR detection.

Little was known about the β-lactam resistance profile of *H. haemolyticus*, a respiratory commensal commonly mis-identified as *H. influenzae* in the diagnostic laboratory. As a result, Chapter 4 examined the phenotypic and genotypic characteristics of β-lactam resistance mechanisms in a large collection of *H. haemolyticus* and *H. influenzae* isolates, collected during the GROMIT study using a well defined patient population. The prevalence and mechanisms of β-lactam resistance were identified to be similar for both bacterial species, with 13.1% of *H. haemolyticus*, and 15.7% of *H. influenzae* isolates harbouring the TEM-1 β-lactamase (with the same replicon and promoter types commonly reported in *H. influenzae* identified in *H. haemolyticus*), whilst 31.0% of both *H. haemolyticus* and *H. influenzae* isolates were positive for the N526K BLNAR-defining substitution. Further analysis of the *ftsI* gene encoding PBP3 in these N526K-positive BLNAR isolates revealed that some of the commonly recognized BLNAR-associated substitutions in *H. influenzae* form part of the baseline PBP3 sequence in *H. haemolyticus*. This suggests that respiratory isolates of *H. haemolyticus* might possibly represent a significant reservoir for β-lactam resistance determinants in co-localised *H. influenzae*.

Examination of the *ftsI* gene sequences of the *H. haemolyticus* isolates from Chapter 4 revealed some differences that might interfere with the SNP-based PCR assays of Hasegawa et al. (2003) that were previously evaluated in Chapter 3 using only *H. influenzae* isolates. When evaluated against a panel of susceptible (N526) and resistant (N526K) isolates of *H. influenzae* and *H. haemolyticus* in Chapter 5, the primer set (PBP3-S) designed to amplify N526-positive (BLNAS) isolates performed well for the identification of susceptible *H. influenzae* isolates. However it failed to

amplify any *H. haemolyticus* isolates, irrespective of their N526/N526K status, due to a species-specific sequence variation in the forward primer-binding region. The discovery of this PCR limitation is significant, as these primers are frequently used in respiratory surveillance studies where *H. haemolyticus* is often mis-identified as *H. influenzae*, and will result in the mis-categorisation of susceptible *H. haemolyticus* isolates as low-BLNAR isolates of *H. influenzae.* A new PCR primer set was therefore developed to overcome this limitation and was 100% sensitive and specific for the separation of N526 isolates (by amplification) from N526K-positive isolates (which fail to amplify) of both species. This is an important new tool for the surveillance of the N526K-positive BLNAR genotype in XV-dependent *Haemophilus* species commonly encountered in the diagnostic laboratory.

Chapter 6 explores the main observation made in Chapter 4, that some of the BLNAR-associated substitutions reported in N526K-positive isolates of NTHi, appear to form part of the baseline PBP3 genotype in susceptible isolates of *H. haemolyticus*. The *ftsI* gene sequences from 100 clinical isolates, including susceptible (N526) and resistant (N526K) *H. influenzae* and *H. haemolyticus* isolates, were examined using a range of bioinformatic approaches for evidence of inter-species recombination events. Mosaic *ftsI* gene sequences were identified in 33% of the isolates tested and shown to represent inter-species recombination events. All recombination events occurred in N526K-positive isolates of either species and frequently resulted in the horizontal transfer of only partial *ftsI* gene fragments. There was no evidence to support the horizontal transfer of the entire *ftsI* gene among the clinical isolates *in vivo*.

Chapter 7 extended on the work of Chapter 6 using an *in vitro* approach. Transformation experiments, using reference recipients and fully characterised N526K-positive isolates of *H. influenzae* and *H. haemolyticus* as donors, were performed to investigate potential inter- and intra-species *ftsI* recombination events. Both inter- and intra-species recombination of the *ftsI* gene frequently occurred in *H. influenzae* and *H. haemolyticus* isolates, and resulted in the formation of mosaic *ftsI* genes that carry the N526K-positive resistance genotype.

In summary, the major findings of this thesis are that a widely used SNP-based PCR algorithm is unreliable for N526K-positive BLNAR detection because of a previously unrecognized SNP encoding the N526K substitution, and because of *ftsI* sequence divergence with *H. haemolyticus* that might mis-identify as *H. influenzae*. As a result, a new SNP-based PCR algorithm was developed and shown to be 100% sensitive and specific for detection of the N526K substitution. Additionally, this thesis presents for the first time the phenotypic and genotypic β-lactam susceptibility profiles of *H. haemolyticus* isolates, and highlights the potential role *H. haemolyticus* plays in the emergence and dissemination of β-lactam resistance determinants in *H. influenzae*. Finally, this thesis has characterised homologous (inter- and intra-species) recombination events involving the *ftsI* gene in both *in vivo* and *in vitro* models. Such *ftsI* recombination events were shown to occur frequently between *H. influenzae* and *H. haemolyticus* isolates, and frequently resulted in the formation of mosaic *ftsI* genes that contribute to the dissemination and diversification of the N526K-positive resistance mechanism in *Haemophilus* species.

Table of Contents

List of Tables

List of Figures

List of Abbreviations

aa	amino acid
AECB	Acute exacerbations of chronic bronchitis
aLRTIs	Acute lower respiratory tract infections
AMC	Amoxicillin-clavulanate
AMP	Ampicillin
AMOX	Amoxicillin
AOM	Acute otitis media
AST	Antimicrobial susceptibility testing
BLNAS	β-lactamase-negative ampicillin-susceptible
BLPAR	β-lactamase-positive ampicillin-resistant
BLNAR	β-lactamase-negative ampicillin-resistant
BLPACR	β-lactamase-positive amoxicillin-clavulanate-resistant
BSAC	British Society for Antimicrobial Chemotherapy
bp	base pair
CEC	Cefaclor
CLSI	Clinical and Laboratory Standards Institute
COPD	Chronic obstructive pulmonary disease
CTX	Cefotaxime
DD	Disc diffusion
DNA	Deoxyribonucleic acid
EUCAST	European Committee on Antimicrobial Susceptibility Testing
GROMIT	Genetics of Recurrent Otitis Media and Immunology in Toddlers
HGT	Horizontal gene transfer
Hib	*Haemophilus influenzae* type b
HTM	Haemophilus test medium
MIC	Minimum inhibitory concentration
MLST	Multi-locus sequence typing
NTHi	Non-typeable *Haemophilus influenzae*
ORF	Open reading frame
PBP	Penicillin-binding protein
PBP3	Penicillin-binding protein 3
PCR	Polymerase chain reaction
PFGE	Pulse field gel electrophoresis
PSG	Phosphate-buffered saline glucose
RTIs	Respiratory tract infections
sBHI	Supplemented brain heart infusion
SNP	Single nucleotide polymorphism
STs	Sequence types
URTI	Upper respiratory tract infection
U.S	United States
USSs	Uptake signal sequences

Chapter 1.0 – Summary of Thesis

1.1 Background

Respiratory tract infections (RTIs) such as acute sinusitis, acute otitis media (AOM), acute and chronic bronchitis, as well as chronic obstructive pulmonary disease (COPD), are one of the most common infections for which antibiotics are currently prescribed in Australia, and these infections are commonly caused by the bacterial pathogen *Haemophilus influenzae*. Successful treatment of these infections is achieved through the prompt prescription of the β-lactam antibiotics amoxicillin, amoxicillin-clavulanate, cefaclor or cefuroxime. However, isolates of *H. influenzae* are increasingly becoming resistant to the aforementioned antibiotics, leading to a significant rate of therapeutic failure estimated to impose a very significant financial burden on the Australian Health Care System.

In *H. influenzae,* resistance to β-lactam antibiotics is mediated through either the production of β-lactamase enzymes that degrade the antibiotic, or the production of an altered penicillin-binding protein 3 (PBP3) that has a reduced binding affinity for antibiotics of the β-lactam class. In some cases, although rare, both resistance mechanisms may be present simultaneously in a single isolate.

The genotypic and phenotypic characteristics of β-lactamase mediated resistance have been extensively studied in this organism, with enzyme, promoter and replicon type, as well as epidemiology well described. In addition, β-lactamase production is easily detected in the diagnostic laboratory such that its clinical significance is clearly defined. In contrast, although the nature of the mutations in

the *ftsI* gene and associated amino acid substitutions in the encoded PBP3 have been extensively reported, the laboratory detection of this important resistance mechanism is less clearly defined. Furthermore, the epidemiology and dissemination of this resistance mechanism is currently poorly understood. As a result, the research presented in this thesis aims to address the specific gaps in the knowledge surrounding our understanding of laboratory detection, epidemiology and prevalence of β-lactamase-negative ampicillin-resistance in *H. influenzae* and its close phylogenetic relative *Haemophilus haemolyticus*.

1.2 Chronology of Works and Thesis Organisation

This chapter of the thesis (**Chapter 1**) comprises a general introduction into the overall themes, rationale of the thesis along with a note on the chronology of the laboratory works conducted.

Chapter 2 presents a review of the current literature that summarises the background information on the main themes of the thesis, and includes a general overview of *H. influenzae* as a human pathogen (bacteriology, pathogenesis, and antibiotic therapy trends), and a detailed review of the antibiotic resistance mechanisms currently recognised to mediate resistance to β-lactam antibiotics in *H. influenzae*. Additionally, a review of the literature underpinning altered penicillin-binding protein mediated resistance is discussed with respect to epidemiology, phenotypic and genotypic characteristics and detection methods currently used in the diagnostic laboratory. Finally, this chapter finishes with the specific aims of the thesis.

Chapter 3 presents an edited version of the first manuscript, which aimed to evaluate current PCR based approaches used in the laboratory detection of BLNAR mediated resistance. In this study it was hypothesised that, as current PCR assays were deigned to target N526K encoded by the AAG SNP, these assays would perform poorly in the detection of N526K encoded by the more recently recognised AAA SNP at the same position of the encoding *ftsI* gene (position, 1576-1578 bp).

An unedited version of this manuscript was published as:

Witherden EA, Kunde D, Tristram SG (2012). An evaluation of SNP-based PCR methods for the detection of β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *J Infect Chemother*; 18:451-5. doi:10.1007/s10156-011-0356-5.

The novel findings from this study were that only the "sensitive" primers, designed to amplify susceptible β-lactamase-negative ampicillin-susceptible (BLNAS) isolates, performed well in the separation of BLNAS and BLNAR populations. Whilst in assays where BLNAR isolates were identified through amplification, BLNAR isolates encoded by the AAA SNP would go undetected.

Chapter 4 presents an edited version of the second manuscript, which aimed to characterise the phenotypic and genotypic characteristics of β-lactam resistance mechanisms in isolates of *H. influenzae* and the closely related respiratory commensal *H. haemolyticus.* The hypothesis of this study tested whether the same mechanisms mediating β-lactam resistance in *H. influenzae* were also present in isolates of *H. haemolyticus*, and whether the prevalence of these resistance mechanisms occurred at similar frequencies.

An unedited version of this manuscript was published as:

Witherden EA, and Tristram SG (2013). Prevalence and mechanisms of β-lactam resistance in *Haemophilus haemolyticus*. *J Antimicrob Chemother*; 68 (5): 1049-1053. doi:10.1093/jac/dks532.

The novel findings from this study were that *H. haemolyticus* harboured the same resistance mechanisms as *H. influenzae*, with resistance occurring at similar prevalence's between the two species. Furthermore, it was identified that some of the PBP3 substitutions commonly identified in BLNAR isolates of *H. influenzae* formed part of the baseline PBP3 genotype in susceptible *H. haemolyticus* isolates.

Based on the finding of BLNAR mediated resistance in *H. haemolyticus* isolates in Chapter 4, **Chapter 5** presents an edited version of the third manuscript that investigated the performance of PCR assays for detecting BLNAR mediated resistance in both *H. influenzae* and *H. haemolyticus*.

An unedited version of this manuscript was published as:

Witherden EA, Kunde D, Tristram SG (2013). PCR screening for the N526K substitution in isolates of *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother* 2013; 68 (10):2255-2258. doi: 10.1093/jac/dkt189.

The novel findings from this study were that susceptible isolates of *H. haemolyticus* with the BLNAS genotype fail to amplify due to a discrepancy in the forward primer-binding region between *H. influenzae* and *H. haemolyticus* species. This is a concern, as these PCR assays are routinely used in respiratory surveillance studies, where *H. haemolyticus* is often mis-identified as NTHi, and this leads to the mis-categorisation of susceptible *H. haemolyticus* isolates as low-BLNAR isolates of *H. influenzae*. As a result, new PCR primers were designed to overcome this

shortcoming, producing a significant new tool for the surveillance of BLNAR mediated resistance in respiratory *Haemophilus* isolates.

Chapter 6 presents an edited version of the fourth manuscript which details the role genetic recombination of the *ftsI* gene plays in the diversification and dissemination of BLNAR genotypes in both *H. influenzae* and *H. haemolyticus*. The hypothesis tested was that *ftsI* genes could be exchanged between *H. influenzae* and *H. haemolyticus* isolates co-localised in the respiratory tract, forming mosaic *ftsI* genes that encode BLNAR mediated resistance.

An unedited version of this manuscript is currently in press as:

Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A (2014). The role of inter-species recombination of the *ftsI* gene on the dissemination of altered penicillin-binding protein 3 mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother*. In Press. doi: 10.1093/jac/dku022.

The novel findings from this study were that mosaic *ftsI* gene structures that had formed through *in vivo* homologous recombination events were identified, and these involved the recombination of the *ftsI* genes from *H. influenzae* and *H. haemolyticus* isolates. In all cases mosaic *ftsI* gene structures were identified in N526K-positive BLNAR isolates of both species, suggesting that horizontal gene transfer and recombination of *ftsI* genes from *H. influenzae* and *H. haemolyticus* has contributed to the dissemination and diversification of BLNAR genotypes.

Chapter 7 presents a manuscript that builds on the work of chapter 6. Here we evaluated and characterised *ftsI* gene recombination events using *in vitro* transformation experiments. The novel findings from this study were that exchange of resistant *ftsI* genes most often resulted in mosaic *ftsI* gene structures, and this

occurred in both an inter- and intra-species manner. A manuscript based on this chapter will soon be submitted for publication.

Chapter 8 presents a general discussion and the concluding remarks regarding the novel findings from the studies undertaken in the thesis. This chapter also addresses the potential limitations of the studies conducted and the future directions of further studies into BLNAR mediated resistance in *Haemophilus* species.

Finally, as this thesis comprises a number of published manuscripts, that have been reformatted to fit the style requirements for a thesis submitted at the University of Tasmania, there are some important points to note, 1). All referencing styles were amended to the author-date style of Diagnostic Microbiology and Infectious Diseases, and a combined reference list is presented at the end of the thesis in Chapter 9, 2). Supplementary data that accompanies the published manuscripts online has been included at the end of each individual manuscript chapter where applicable, 3). There is some repetition of abbreviations and terms between chapters, such that chapters may be read as stand alone manuscripts.

Chapter 2.0 - General Introduction and Review of the Literature

2.1 *H. influenzae* as a Human Pathogen

The *Haemophilus* genus belongs to the bacterial family *Pasteurellaceae,* and consists of 14 closely related species that are indigenous to the mucosal linings of the oropharynx, nasopharynx and genital tract of mammals (Winn, 2006). Of the nine *Haemophilus* species that colonize humans, *H. influenzae* is the most clinically important as it is considered a significant opportunistic pathogen in a number of invasive and non-invasive infections (Winn, 2006; Kuhnert and Christensen, 2008).

2.1.1 Bacteriology of *H. influenzae*

H. influenzae are small, fastidious, non-motile gram-negative bacilli that require the presence of the blood derived co-factors hemin (X factor) and nicotinamide-adenine-dinucleotide (NAD – V factor) for *in vitro* growth (Winn, 2006). On culture, isolates of *H. influenzae* are non-haemolytic facultative anaerobes that grow optimally at 37^{O}C in 5-7% CO_2 (Winn, 2006). On the basis of biochemical testing all isolates of *H. influenzae* can be conveniently categorised into one of seven clinically important biotypes (I-VII) which are loosely linked to different patterns of virulence, pathogenicity and epidemiology (Kilian, 1976; Krieg, 2001; Winn, 2006).

The pathogenicity of *H. influenzae* is intrinsically linked to the presence of virulence factors that aid in adherence, colonization, invasion and in some isolates, capsular

production (Herbert et al., 2003; Winn, 2006). Of the numerous virulence factors in *H. influenzae*, the ability to produce a polysaccharide capsule is the most clinically important because it facilitates bacterial survival, particularily in the blood stream of which may lead to invasive infections. As a result isolates can be conveniently categorised into encapsulated (typeable) and non-encapsulated (non-typeable) based on the presence or absence of this capsule (Herbert et al., 2003; Winn, 2006).

2.1.1.1 Encapsulated *H. influenzae*

Encapsulated isolates produce mucoid colonies when cultured, and can be serologically typed on the basis of antigenically distinct polysaccharide capsules (a through f) (Pittman, 1931). Most encapsulated isolates belong to *H. influenzae* biotype I, and are often involved in serious invasive infections, including cases of meningitis, pneumonia, bacteraemia, epiglottitis, cellulitis and septic arthritis (Kilian, 1976; Winn, 2006). *H. influenzae* serotype b (Hib) is the most clinically significant serotype as it is frequently involved in serious infections in children, particularly cases of paediatric meningitis and pneumonia in children under 5 years of age (Herbert et al., 2003; Winn, 2006).

Prior to the introduction of the Hib conjugate vaccine, Hib meningitis accounted for 3 million cases of serious illness, resulting in 50-60 deaths per 100,000 children under the age of 5 in the United States (U.S), and 25 and 35 deaths per 100,000 children, in Australia and Europe, respectively (Peltola, 2000; Watt et al., 2009). Today the Hib conjugate vaccine forms part of the routine vaccination schedule in 108 countries and this has resulted in a significant reduction in the number of

serious cases of meningitis and pneumonia caused by Hib (Watt et al., 2009). In contrast to the low incidence of Hib meningitis cases now reported in vaccinated countries, (i.e., < 1 per 100, 000 children under the age of 5 in the U.S, Australia and Europe), Hib still accounts for a significant proportion of 'preventable' infant morbidities and mortalities in those countries where the Hib vaccination is yet to be introduced (Peltola, 2000; Hasegawa et al., 2004; Watt et al., 2009; McIntyre et al., 2012; Ubukata et al., 2013).

Due to the success of Hib immunisation programs other encapsulated serotypes are now more frequently associated with opportunistic invasive infections in both paediatric and adult populations (Cerquetti et al., 2000). In particular, *H. influenzae* serotypes a, d and f have been linked to pneumonia and bacteraemia in immunocompromised adults, whilst serotype c has been isolated in cases of neonatal sepsis (Slack et al., 1998; Cerquetti et al., 2000; MacNeil et al., 2011; Fischer, 2014).

2.1.1.2 Non-encapsulated *H. influenzae*

Non-encapsulated *H. influenzae* do not produce a polysaccharide capsule, are non-mucoid and non-haemolytic on culture, and are normal commensal flora of the human nasopharynx (Winn, 2006). Similar to encapsulated isolates, nasopharyngeal colonisation with non-typeable *H. influenzae* (NTHi) begins shortly after birth, and is influenced by a number of interrelated factors including locality, age, childcare centre attendance, and antibiotic therapy (Herbert et al., 2003; King, 2012). Even though NTHi isolates are usually considered less pathogenic than their encapsulated

counterparts, they have been implicated in a number of localised RTIs (Foxwell et al., 1998; Murphy, 2003; Murphy et al., 2009).

In the developed world NTHi is considered an important aetiological agent in acute and chronic RTIs in both paediatric and adult populations (Murphy et al., 2009; King, 2012). NTHi is currently responsible for 25-35% of all AOM cases (a significantly higher proportion of recurrent cases), and is responsible for one third of all reported sinusitis infections (Herbert et al., 2003). NTHi is also frequently involved in cases of community-acquired pneumonia (CAP), paediatric conjunctivitis, and acute exacerbations of both chronic bronchitis (AECB), and COPD (Herbert et al., 2003). β-lactam antibiotics are currently recommended as empiric therapy used in the treatment of many of these infections, and includes the prompt prescription of amoxicillin, amoxicillin-clavulanate or cefaclor (Herbert et al., 2003; Murphy et al., 2009).

Since the incidence of invasive infections caused by Hib has decreased following widespread Hib vaccination programs, NTHi has become increasingly recognised as an opportunistic invasive pathogen, which has been isolated in cases of meningitis and bacteraemia in both immunocompromised children and adults with underlying medical conditions (Murphy et al., 2009).

2.1.2 β-lactam Therapy for *H. influenzae* Infections

The choice and likely success of antibiotic therapy for treatment of infections caused by *H. influenzae* depends on the age of the patient, the nature of the

infection and the presence or likelihood of resistance mechanisms. In most cases antibiotics from the β-lactam class are the empiric therapy of choice, which includes the prescription of both oral and parental agents (Antibiotic Expert Group, 2010). The oral β-lactam antibiotics amoxicillin, amoxicillin-clavulanate, cefaclor and cefuroxime are currently commonly prescribed in Australia, the U.S and Europe to treat non-invasive respiratory infections caused by *H. influenzae* (Hoberman et al., 2002; Murphy, 2003; Antibiotic Expert Group, 2010). In contrast, parental β-lactam agents such as the extended-spectrum cephalosporin's cefotaxime and ceftriaxone or carbapenems such as meropenem are usually reserved for the treatment of invasive life-threating infections caused by *H. influenzae* (Antibiotic Expert Group, 2010).

It is important to note that the prescribing practices of Australia, the U.S and Europe differ greatly from those seen in Asian countries (Japan, Korea and China), where they favour oral cephalosporin's (i.e. cefaclor, cefdinir, cefditoren) (Hasegawa et al., 2003). The use of different empirical β-lactam agents is believed to contribute to the different levels of β-lactam resistance seen in *H. influenzae* between these countries (Hoberman et al., 2002; Murphy, 2003).

2.1.3 Mechanisms of β-lactam Resistance in *H. influenzae*

In *H. influenzae,* resistance or reduced susceptibility to β-lactam antibiotics is mediated by either the production of β-lactamase enzymes that degrade and inactive β-lactam antibiotics, or by the presence of altered penicillin-binding proteins (PBPs) that have lowered binding affinities for β-lactam antibiotics

(Ubukata et al., 2001; Tristram et al., 2007). To date, neither efflux pumps or altered cell permeability have been shown to contribute to β-lactam resistance in *H. influenza*e, although, there has been a single report of a mutant AcrAB efflux pump contributing to higher levels of ampicillin resistance in a small number of North American BLNAR isolates (Kaczmarek et al., 2004).

As the subsequent chapters of this thesis focus on altered PBP mediated resistance, a brief overview of the current β-lactam resistance mechanisms recognised in *H. influenzae* follows in this section. Table 2.1 summarises the current global prevalence of β-lactam resistance in *H. influenzae* by mechanism where; isolates that harbour no resistance mechanism and are ampicillin susceptible are termed β-lactamase-negative ampicillin-susceptible (BLNAS); isolates that are ampicillin resistant due to the production of β-lactamase enzymes are termed β-lactamase-positive ampicillin-resistant (BLPAR); isolates resistant to ampicillin due to altered PBPs are termed β-lactamase-negative ampicillin-resistant (BLNAR); and, isolates that harbour both β-lactamase enzymes and altered PBPs are termed β-lactamase-positive amoxicillin-clavulanate-resistant (BLPACR).

2.1.3.1 β-lactamase Enzymes

β-lactamase production was first reported in *H. influenzae* in the late 1970's with the discovery of the TEM-1 β-lactamase and later the ROB-1 β-lactamase (Medeiros and O'Brien, 1975; Rubin et al., 1981). Both β-lactamases are class A serine plasmid-mediated β-lactamases that share similar substrate hydrolysis profiles that infer

resistance to the aminopenicillins, ampicillin and amoxicillin (Karlowsky et al., 2000).

The phenotype of BLPAR isolates is characterised by a raised ampicillin MIC, usually in the range of ≥ 64 μg/mL, that is well above the ampicillin resistant breakpoint of ≥ 4 μg/mL according to the Clinical and Laboratory Standards Institute (CLSI) susceptibility guidelines (CLSI, 2013).

In a diagnostic setting this raised ampicillin MIC needs to be coupled with a positive nitrocefin disc test for confirmation of β-lactamase production, whilst molecular assays must be employed if differentiation of the β-lactamase type (TEM-1 or ROB-1) is desired (Tristram and Nichols, 2006; Tristram et al., 2012; CLSI, 2013).

2.1.3.1.1 Prevalence

β-lactamase production is the most common mechanism of resistance in *H. influenzae* with on average 15-20% of *H. influenzae* isolates producing β-lactamase enzymes (Doern et al., 1999). Although, the prevalence of β-lactamase production had plateaued in *H. influenzae* at around 20%, the absolute prevalence varies greatly by geographical location; Australia (27.5%); Japan (8.5%); South Korea (64.7%); Canada (19.6%); U.S (25.7%), respectively (Hoban and Felmingham, 2002).

Molecular analysis of the β-lactamase enzymes produced by *H. influenzae* demonstrated that 90-95% of all BLPAR isolates harbour the TEM-1 β-lactamase, whilst the remaining 5-10% produce the ROB-1 type β-lactamase (Farrell et al., 2005). To date only one BLPAR isolate has been reported to simultaneously produce both a TEM-1 and ROB-1 β-lactamase (Farrell et al., 2005).

Table 2.1 Worldwide prevalence of β-lactam resistance mechanisms in clinical *H. influenzae* isolates by origin, source of isolation and method of study.

Origin and source of Isolates[a]	Study[b]	(n)	Percent per resistance class (%)[c]			
			BLNAS	BLPAR	BLNAR	BLPACR
Japanese						
Meningitis	Hasegawa et al. (2004)[+]	395	29.1	15.4	44.6	10.9
	Hasegawa et al. (2006)[+]	621	25.0	11.0	52.7	11.4
RTIs	Hasegawa et al. (2003)[+]	296	55.1	3.0	39.5	2.4
	Kubota et al. (2006)[++]	160	68.1	15.0	16.9	0.0
	Hotomi et al. (2007)[+]	264	33.0	0.8	65.2	1.1
	Qin et al. (2007)[+]	163	76.7	9.2	13.5	0.6
	Ohkoshi et al. (2008)[+]	457	47.3	3.1	46.4	3.3
	Niki et al. (2011)[++]	187	54.0	5.3	40.6	0.0
Paediatric AOM	Sakai et al. (2005)[+]	150	70.7	4.7	23.3	1.3
	Kishii et al. (2010)[+]	191	39.8	0.5	56.0	3.7
European						
RTIs	Marco et al. (2001)[++]	1730	67.6	23.1	9.3	0.0
	Fluit et al. (2005)[++]	915	78.6	12.2	9.2	0.0
	Jansen et al. (2006)[++]	578	83.6	7.6	8.8	0.0
	Barbosa et al. (2011)[++]	2160	63.3	24.2	12.5	0.0
RTIs+Meningitis	Garcia-Cobos et al. (2007)[+]	354	17.8	15.8	56	10.4
Korean						
RTIs	Kim et al. (2007)[+]	229	23.6	38.9	29.3	8.3
	Bae et al. (2010)[+]	540	41.5	47.2	6.1	5.2
Australian						
	Robson et al. (2006)[++]	1822	69.6	21.9	8.5	0.0
United States						
RTIs	Doern et al. (1997)[++]	1537	61.0	35.3	2.5	1.1
	Karlwosky et al. (2002)[++]	1434	71.1	28.2	0.6	0.1
	Hasegawa et al. (2003)[+]	100	46.0	36.0	13.0	5.0
Vietnam						
Paediatric aLRTIs	Gotoh et al. (2008)[+]	37	21.6	45.9	16.2	16.2

[a] RTIs, respiratory tract infections; Paediatric AOM, Paediatric acute otitis media; Paediatric aLRTIs, Paediatric acute lower respiratory tract infections.

[b] Data from the aforementioned studies. [+], Designates the use of genetic testing for identification of resistance mechanisms (PCR algorithm, or individual PCR and *ftsI* gene sequencing); [++], designates phenotypic descriptors used for identification of resistance mechanisms (nitrocefin for β-lactamase production; BLNAR defined as ampicillin resistant (MIC ≥ 4 μg/mL); BLPACR, defined by the presence of ampicillin and amoxicillin-clavulanate resistance (amoxicillin-clavulanate MIC ≥ 8 μg/mL). Note that some studies included ampicillin or amoxicillin intermediate isolates (MIC = 2 μg/mL) in their BLNAR definitions.

[c] BLNAS, β-lactamase-negative ampicillin-susceptible; BLPAR, β-lactamase-positive ampicillin-resistant; BLNAR, β-lactamase-negative ampicillin-resistant; BLPACR, β-lactamase-positive amoxicillin-clavulanate-resistant.

The *bla*$_{TEM}$ gene encodes the TEM-1 β-lactamase, and is located on either large integrative conjugative elements (ICEs) or small non-conjugative plasmids (Leaves et al., 2000; Tristram et al., 2007; Fleury et al., 2013). Molecular analysis of these *bla*$_{TEM}$ genes has revealed three distinct TEM-1 promoter types; Pa/Pb, Pdel and Prpt, that are associated with a C32T mutation, a 135 bp deletion and a 54 bp repeat in the promoter region of *bla*$_{TEM}$, respectively (Tristram and Nichols, 2006; Tristram et al., 2012). Other TEM-1 promoter variants have also been described, although they are less routinely encountered (Garcia-Cobos et al., 2008a).

In contrast, ROB-1 β-lactamases are encoded by the *bla*$_{ROB}$ gene and are found only on small non-conjugative plasmids (Brunton et al., 1983). Evidence suggests that the diversity between the location of these two genes and the highly transmissible nature of ICEs among *H. influenzae* has contributed to the higher prevalence of the TEM-1 β-lactamase reported in *H. influenzae* (Leaves et al., 2000).

2.1.3.1.2. Clinical Significance

The presence of either the TEM-1 or ROB-1 β-lactamase infers resistance to both ampicillin and amoxicillin but not to amoxicillin-clavulanate (Farrell et al., 2005; Tristram et al., 2007). TEM-1 producing isolates currently remain susceptible to cefaclor (a second-generation cephalosporin commonly used to treat AOM infections), whilst many ROB-1 producing isolates efficiently hydrolyse cefaclor, inferring cefaclor resistance (Karlowsky et al., 2000; Farrell et al., 2005). Although the association between the presence of ROB-1 and raised cefaclor MICs was originally attributed to possible mutations in the *bla*$_{ROB}$ gene causing altered ROB-1 expression or activity, Tristram and colleagues (2010) more recently linked the

raised cefaclor MICs in these isolates to the coexpression of alterations in PBP3 (Karlowsky et al., 2000; Molina et al., 2003; Tristram et al., 2010).

Isolates with either of these β-lactamase enzymes currently remain susceptible to extended spectrum cephalosporin's and carbepenems (Antibiotic Expert Group, 2010).

2.1.3.2 Altered Penicillin-Binding Protein 3

Non-β-lactamase mediated resistance was first described in *H. influenzae* in 1974, and has since been linked to either an R517H or N526K substitution in penicillin-binding protein 3 (PBP3) (Thornsberry and Kirven, 1974; Ubukata et al., 2001). These amino acid substitutions are the result of specific missense mutations at base pair (bp) positions 1549 to 1551 (for R517H) and 1578 to 1581 (for N526K) of the encoding *ftsI* gene, and are responsible for the reduced binding affinity of PBP3 for β-lactam antibiotics (Ubukata et al., 2001). In BLNAR isolates, alterations to PBP3 frequently infer low-level resistance to ampicillin and amoxicillin, and reduced susceptibility to amoxicillin-clavulanate and cephalosporin's (particularly cefaclor, cefuroxime and cefotaxime) (Doern et al., 1997; Karlowsky et al., 2000; Ubukata et al., 2001; Dabernat et al., 2002; Osaki et al., 2005; Garcia-Cobos et al., 2007; Garcia-Cobos et al., 2008a).

It should be noted that there is currently no consensus definition as to what defines a "BLNAR" isolate, and this makes detection, interpretation of prevalence, and clinical significance difficult. For example, BLNAR isolates can either be defined

phenotypically by ampicillin MIC (although various resistance or non-susceptibility breakpoints are currently used), or genotypically by SNP PCR or sequencing to detect key mutations in the *ftsI* gene. As the complexities of BLNAR definitions and detection methods are discussed in detail in subsequent sections of this review, only a general overview of the major findings in regard to prevalence and clinical significance follow.

2.1.3.2.1 Prevalence

Overall the prevalence of BLNAR mediated resistance has risen significantly over the past decade, from 2.5% in 1997 to 56% in 2010 (Data from Table 2.1). In surveillance studies recently conducted throughout Australia, Europe and the U.S, BLNAR isolates were reported to account for 10-15% of all *H. influenzae* isolates when phenotypic BLNAR descriptors (such as ampicillin MICs of ≥ 2 µg/mL or ≥ 4 µg/mL) are used for detection (Karlowsky et al., 2002; Jansen et al., 2006; Robson, 2006; Jansen et al., 2008). In contrast, an alarmingly higher prevalence has been noted across Asia, particularly when genotypic descriptors (such as SNP PCR for N526K or N526K and S385T) are used for BLNAR detection, such that anywhere from 13-65% of Japanese and Korean *H. influenzae* isolates are reported to harbour the BLNAR genotype, respectively (Hotomi et al., 2007; Kim et al., 2007). More specifically, in a 2010 study of 191 Japanese *H. influenzae* isolates isolated from paediatric patients suffering AOM, the prevalence of BLNAR was reported to be 56% (107/191) which is of alarming concern when compared to the prevalence of 23% (35/150) reported in the same patient population, less than 5 years earlier (Sakai et al., 2005; Kishii et al., 2010).

2.1.3.2.2. Clinical Significance

Although there is currently a lack of consensus surrounding the definition, detection and clinical significance of BLNAR isolates globally, there has been some documented reports of therapeutic failure when patients infected with such isolates are treated with β-lactam antibiotics (Markowitz, 1980; Rubin et al., 1981; van der Ploeg et al., 2008). Ampicillin and cefuroxime treatment failure have been documented in cases of meningitis caused by BLNAR isolates of both NTHi and Hib, respectively (AMP MIC of 6.25 μg/mL; CXM MIC of 4-8 μg/mL) (Markowitz, 1980; Mendelman et al., 1990a). Amoxicillin therapy failure has been reported in an NTHi isolate with low-level resistance to amoxicillin (AMOX MIC of 2 μg/mL) isolated from a child suffering with vertebral osteomyelitis (van der Ploeg et al., 2008).

Current CLSI guidelines recommend that all clinical BLNAR isolates be reported as resistant to ampicillin, amoxicillin, cefaclor, cefuroxime, as well as amoxicillin-clavulanate, irrespective of *in vitro* susceptibilities (CLSI, 2013). Although it is not clear, this recommendation probably only applies to those BLNAR isolates with ampicillin MICs ≥ 4 μg/mL (high-BLNAR), as these are the current conditions under which CLSI recognize a BLNAR isolate (CLSI, 2013). Consequently, confusion surrounding susceptibility and clinical significance will occur in those instances where *ftsI* gene sequencing and low-BLNAR definitions have been used to identify BLNAR isolates (Tristram et al., 2007).

2.1.3.3 Both Mechanisms

Isolates of *H. influenzae* that simultaneously contain β-lactamase enzymes and altered PBP3's are currently infrequently observed. These BLPACR isolates were first described at a time when altered PBP3 (BLNAR) mediated resistance was uncommon and not well understood (Doern et al., 1997; Tristram et al., 2007). Originally the mechanism of resistance in BLPACR isolates was thought to be due to the hyper production of the TEM-1 β-lactamase, or the production of a novel β-lactamase resistant to amoxicillin-clavulanate (Doern et al., 1997). As a result BLPACR isolates were classified as any β-lactamase-positive isolate that was resistant to both ampicillin (MIC ≥ 4 µg/mL) and amoxicillin-clavulanate (MIC ≥ 8/4 µg/mL), according to CLSI breakpoints (Tristram et al., 2007; CLSI, 2013).

When the first BLPACR isolates were molecularly characterised in 2003, it was identified that BLPACR isolates had much higher amoxicillin MICs than their BLNAR counterparts with identical PBP3 substitutions (BLNAR, 8-32 µg/mL; BLPACR, ≥ 64 µg/mL) (Matic et al., 2003). Interestingly, the MICs to amoxicillin-clavulanate and the cephalosporin's cefaclor and cefuroxime remained similar between the two resistant populations (Matic et al., 2003). This suggested that the activity of the β-lactamase in BLPACR isolates was specific to ampicillin, and that the resistance to amoxicillin-clavulanate was most likely conferred by the alterations in PBP3, a theory subsequently confirmed by Tristram and colleagues (2010) (Matic et al., 2003; Tristram et al., 2010).

2.1.3.3.1 Prevalence

Estimating the global prevalence of BLPACR isolates is compromised by the difficulty in detecting them. This is because most isolates that produce both a β-lactamase and altered PBP3s, actually have amoxicillin-clavulanate MICs in the susceptible range and therefore go undetected when phenotypic descriptors are used (Tristram et al., 2007). However, when genotypic methods are used for detection, BLPACR isolates are more easily recognised. Irrespective of the detection method used it is currently generally accepted that the overall prevalence of BLPACR in *H. influenzae* remains low compared to BLPAR and BLNAR mediated resistance, respectively (Fluit et al., 2005; Hasegawa et al., 2006; Garcia-Cobos et al., 2008a; Barbosa et al., 2011)

Looking specifically at the 1537 *H. influenzae* isolates studied in the U.S only 1.1% (17/1537) were identified to be BLPACR on phenotypic testing (Doern et al., 1997). In contrast, among Japanese studies using genotypic methods for BLPACR detection 11% of isolates (43/395; 71/621) were identified to be BLPACR in two consecutive surveillance studies (Hasegawa et al., 2004; Hasegawa et al., 2006). However, in another Japanese study conducted in 2005, only 1.3% (2/150) of isolates were reported to be BLPACR, even though the same genotypic method of detection was employed (Sakai et al., 2005).

In Europe, 14% (15/108) of French *H. influenzae* isolates, and 20% (47/240) of Portuguese *H. influenzae* isolates have been identified as BLPACR by genotypic methods, although in both studies isolates were originally pre-screened for by reduced susceptibility to β-lactam antibiotics (Dabernat et al., 2002; Barbosa et al., 2011). Interestingly, of the 47 BLPACR isolates identified by genotype in Portugal,

none were phenotypically resistant to amoxicillin-clavulanate according to current CLSI criteria, instead the amoxicillin-clavulanate MICs of the BLPACR isolates ranged from 1-4 μg/mL and this correlates with the findings of other studies, where the majority of BLPACR isolates actually have reduced susceptibility to amoxicillin-clavulanate and not resistance (Dabernat et al., 2002; Tristram et al., 2007; Barbosa et al., 2011).

In the aforementioned studies all BLPACR isolates were identified to be carrying the TEM-1 type β-lactamase. There has only been a single report of a BLPACR isolate harbouring the ROB-1 type β-lactamase and this isolate was susceptible to amoxicillin-clavulanate (1 μg/mL) according to CLSI breakpoints (San Millan et al., 2011).

2.1.3.3.2 Clinical Significance

The presence of β-lactamase enzymes in BLPACR isolates confers high-level resistance to ampicillin and amoxicillin, similar to that seen in BLPAR isolates (Matic et al., 2003). As the effect of the β-lactamase is limited to aminopenicillins, the reduced susceptibility and resistance to amoxicillin-clavulanate and some cephalosporin's is attributed to the presence of alterations in PBP3, such that MICs to these agents are comparable to those in BLNAR isolates with identical PBP3 substitutions (Matic et al., 2003; Barbosa et al., 2011). As a result the same issues surrounding the clinical significance of BLNAR isolates apply here, to BLPACR isolates.

2.2 Evidence for Penicillin-Binding Protein 3 Mediated Resistance

2.2.1 Background History

Non-β-lactamase mediated resistance to ampicillin was first described in *H. influenzae* by Thornsberry and Kirven (1974) when they encountered two ampicillin resistant isolates (AMP MIC of 8 μg/mL) that tested negative for β-lactamase production (Thornsberry and Kirven, 1974). Although numerous attempts were made to induce β-lactamase production in this original study, the mechanism of resistance could not be elucidated. In subsequent years similar BLNAR isolates were reported among clinical isolates of Hib and subsequently NTHi and the mechanism of resistance was hypothesized to be due to either; cell envelope modifications, altered PBPs, altered porin channels, or varied cell wall lipopolysaccharides (LPS) (Markowitz, 1980; Mendelman et al., 1984; Tremblay et al., 1990; Clairoux et al., 1992).

2.2.2 Evidence for PBP3: Binding and Transformation Studies

The mechanism of ampicillin resistance in BLNAR isolates was partially clarified when Parr and Bryan (1984) successfully transformed *H. influenzae* Rd with genomic DNA from a clinical Hib BLNAR isolate (Parr and Bryan, 1984). In that study, the investigators demonstrated a reduction in the binding affinity of PBP3 (formerly, PBP3a and PBP3b) for ampicillin that correlated with a phenotypic increase in ampicillin MIC (Parr and Bryan, 1984). Subsequent transformation studies by Mendelman et al. (1990a) and Clairoux et al. (1992), using other clinical BLNAR

isolates confirmed the involvement of PBP3 in resistance and also excluded altered porin channels and cell envelope modifications as possible mechanisms of resistance (Mendelman et al., 1990b; Clairoux et al., 1992).

Characterisation of the BLNAR isolates studied by Clairoux et al. (1992), identified three distinct levels of ampicillin resistance; group I, ampicillin MIC of 0.5 μg/mL; group II, ampicillin MIC of 2-4 μg/mL; and group III, ampicillin MIC ≥ 8 μg/mL (Clairoux et al., 1992). Using genomic DNA from the aforementioned donor groups Clairoux and colleagues (1992), successfully transformed *H. influenzae* Rd, and showed that the transformants displayed elevated ampicillin MICs similar to their respective donor groups (Clairoux et al., 1992). This suggested that the degree of ampicillin resistance in a given BLNAR isolate was directly attributable to the presence of specific modifications in PBP3 (Clairoux et al., 1992).

In 2001, the molecular mechanism behind BLNAR was clarified (Ubukata et al., 2001). Investigators identified that the chromosomally located *ftsI* gene encoded PBP3 in *H. influenzae*, and demonstrated that transformation of *H. influenzae* Rd with the *ftsI* gene from BLNAR isolates conferred the same level of ampicillin resistance and reduction in PBP3 binding affinity as the respective donor (Ubukata et al., 2001). Furthermore, comparison of *ftsI* gene sequences amplified from 25 Japanese BLNAR isolates and five BLNAS control isolates identified two specific missense mutations in the *ftsI* gene of BLNAR isolates that encoded key amino acid substitutions in the transpeptidase domain of PBP3 (Ubukata et al., 2001). These amino acid substitutions include; an arginine (R) to histidine (H) substitution at position 517 (R517H), and an asparagine (N) to lysine (K) substitution at position

526 (N526K) of PBP3, and these substitutions define the BLNAR genotype today (Ubukata et al., 2001).

2.2.3 PBP3 in *H. influenzae*

PBP3 is a relatively large molecular weight protein (75 kDa) that is involved in the cross-linking of peptidoglycan polymers in the bacterial cell wall (Zapun et al., 2008). Like other integral PBPs, PBP3 consists of three distinct and functionally essential domains; a short cytoplasmic domain, a membrane-spanning domain, and a transpeptidase domain (Zapun et al., 2008).

Within the transpeptidase domain of PBP3 (amino acids 320 to 573) there are three conserved amino acid motifs; Ser-327-Thr-Val-Lys (STVK), Ser-379-Ser-Asn (SSN), and Lys-513-Thr-Gly (KTG), all of which are essential for PBP3 function (Pares et al., 1996). Using the deduced PBP3 amino acid sequences from clinical BLNAR and BLNAS isolates, two authors have constructed three-dimensional (3D) models of PBP3 in *H. influenzae* (Ubukata et al., 2001; Straker et al., 2003). These studies identified that the key BLNAR-defining substitutions (R517H and N526K) are located within the active-site pocket of the mature protein, adjacent to the catalytic serine residue (S327) and although protein modelling is based on a number of assumptions, both groups identified that the presence of the N526K substitution resulted in a conformational change in the active-site pocket of the mature protein (Ubukata et al., 2001; Straker et al., 2003).

2.3 Amino acid Substitutions in PBP3

The advent of readily available *ftsI* gene sequencing has allowed the translated amino acid sequences of PBP3 to be extensively studied. Comparison of the deduced amino acid sequences in PBP3 from BLNAR isolates to that of susceptible reference strain *H. influenzae* Rd has identified the presence of 38 different substitutions in the transpeptidase domain of PBP3 to date. Most BLNAR isolates harbour multiple substitutions in the transpeptidase domain of PBP3, although the absolute number ranges from 3-11 substitutions between individual isolates (Ubukata et al., 2001; Tristram et al., 2007). Interestingly, the exact amino acid residue substituted at any given position appears to be highly conserved among isolates (Ubukata et al., 2001). The contribution of these amino acid substitutions on ampicillin MIC has been extensively studied, such that the substitutions can be classified according to their contribution to β-lactam resistance into: BLNAR-defining or BLNAR-associated (also termed miscellaneous by some authors).

2.3.1 BLNAR-defining Substitutions

The term BLNAR-defining refers to those substitutions that are directly responsible for the reduced susceptibility to ampicillin seen in BLNAR isolates, and includes the R517H and N526K substitutions (Ubukata et al., 2001). In most instances a BLNAR isolate will harbour only one of these two substitutions, either R517H or N526K at a given time (Ubukata et al., 2001; Tristram et al., 2007). Admittedly, there has been a single report of a Korean BLNAR isolate harbouring both the R517H and N526K

substitutions simultaneously, however this isolate did not undergo full molecular characterisation (Kim et al., 2007). Of the two BLNAR-defining substitutions, N526K is the most clinically significant as it is encountered much more frequently than R517H, even though both substitutions give rise to similar increases in ampicillin MIC (Ubukata et al., 2001).

2.3.2 BLNAR-associated Substitutions

Other additional amino acid substitutions have been reported within the transpeptidase domain of PBP3, and these are frequently referred to as BLNAR-associated. These substitutions are commonly reported as additional substitutions that occur concurrently with either R517H or N526K, and are routinely used to sub-categorise BLNAR genotypes (Ubukata et al., 2001; Dabernat et al., 2002). Commonly reported BLNAR-associated substitutions include D350N, S352T, S357N, M377I, S385T, L389F A437S, I449V, G490E, A502V/T, T532S, V547I and N569S (Ubukata et al., 2001; Dabernat et al., 2002; Osaki et al., 2005; Skaare et al., 2010; Barbosa et al., 2011). Of these BLNAR-associated substitutions only M377I, S385T and L389F have been widely studied, as they were originally isolated in BLNAR isolates with relatively high ampicillin MICs (4-8 μg/mL) (Ubukata et al., 2001).

The recent description of some BLNAR-associated substitutions in ampicillin susceptible isolates of *H. influenzae* (in the absence of R517H or N526K) further confuses the clinical significance of these additional substitutions (Garcia-Cobos et al., 2007; Garcia-Cobos et al., 2008a; Barbosa et al., 2011).

2.3.3 Substitutions Identified in PBP3

Table 2.2 summarises the range of amino acid substitutions currently recognised and reported in BLNAR isolates of *H. influenzae* worldwide.

Based on the presence and absence of amino acid substitutions identified in PBP3, BLNAR isolates can be categorised by distinct substitution profiles into Groups (Gp) I, II and III, respectively (Ubukata et al., 2001). Gp I isolates are defined by the presence of the R517H substitution, Gp II isolates by the N526K substitution, and Gp III isolates by the presence of N526K in conjunction with three additional substitutions; M377I, S385T, and L389F. In this original study other additional miscellaneous substitutions including D350N and S357N were also frequently reported (Ubukata et al., 2001).

Dabernat et al. (2002) built on the work of Ubukata et al. (2001) whilst studying the deduced amino acid sequences from 108 French BLNAR isolates. Among the French isolates only Gp I (R517H-positive) and Gp II (N526K-positive) BLNAR's were identified, although the majority of the N526K-positive isolates also harboured additional substitutions at positions 490 and 502 of PBP3 (Dabernat et al., 2002). As a result the N526K-positive BLNAR isolates were further sub-divided into four subgroups (Gp IIa, IIb, IIc, and IId), based on the presence or absence of these two additional substitutions, and are defined as follows; Gp IIa N526K only; Gp IIb N526K and A502V; Gp IIc N526K and A502T; Gp IId N526K and V449I (Dabernat et al., 2002).

Table 2.2 Deduced amino acid substitutions identified in the transpeptidase domain of PBP3 in BLNAR and BLPACR isolates reported in the literature, compared with control strain *H. influenzae* Rd (Kw20).

	bla		Amino acid at position[a]:																						
Strain	**(-)**	**(+)**	**337**	**350**	**352**	**357**	**368**	**377**	**385**	**389**	**437**	**443**	**449**	**490**	**501**	**502**	**511**	**517**	**526**	**528**	**530**	**532**	**547**	**562**	**569**	**586**
Rd			**A**	**D**	**S**	**S**	**A**	**M**	**S**	**L**	**A**	**T**	**I**	**G**	**R**	**A**	**V**	**R**	**N**	**Y**	**T**	**T**	**V**	**V**	**N**	**A**
Ubukata et al. (2001)																										
Gp I	9			N		N		I	T		S							**H**							S	
Gp II	12			**N**		N										V			**K**				I		S	
Gp III	4			**N**		**N**		**I**	**T**	**F**									**K**				I	L	S	
Dabernat et al. (2002)																										
Gp I	7			N		N										T		**H**								
Gp IIa	4	1																	**K**							
Gp IIb	54	2		N		N		I			S			E		**V**			**K**							
Gp IIc	22	3		N			T									**T**			**K**							
Gp IId	6	9											**V**						**K**							
Straker et al. (2003)																										
Gp I	1																A	**H**								
Gp IIa	6		V			N						A			H/L				**K**	H		S				
Gp IIb	3			N				I								**V**			**K**							
Gp IIc	3						T									**T**			**K**							
Hasegawa et al. (2004)																										
I	9																	**H**								
II	2							**I**	**T**	**F**								**H**								
III	7								**T**										**K**							
IV	1							**I**	**T**										**K**							
V	1								**T**	**F**									**K**							
VI	35							**I**	**T**	**F**									**K**							
VII		1							**T**									**H**								
VIII		6						**I**	**T**	**F**									**K**							

[a] Boldface type, all isolates; underline, most isolates; plain text, some isolates; *, amino acid substitution identified at adjacent position.

[b] Depicts deduced amino acid substitutions in PBP3 of cefuroxime resistant isolates. One isolate had a R501L substitution, instead of the R501H substitution previously reported at this position.

	bla		Amino acid at position[a]:																						
	(-)	(+)	337	350	352	357	368	377	385	389	437	443	449	490	501	502	511	517	526	528	530	532	547	562	569	586
Rd			**A**	**D**	**S**	**S**	**A**	**M**	**S**	**L**	**A**	**T**	**I**	**G**	**R**	**A**	**V**	**R**	**N**	**Y**	**T**	**T**	**V**	**V**	**N**	**A**
Fluit et al. (2005)																										
Gp I	1																	**H**								
Gp IIa	3			N										E					**K**		S					
Gp IIb	11			N		N		I						E		**V**			**K**							
Gp IIc	10			N			T									**T**			**K**							
Gp IId	1												**V**						**K**							
Kubota et al. (2006)																										
Gp IIa	7			N		N											A		**K**				I		**S**	S
Gp III	13			**N**	F	**N**		**I**	**T**	**F**									**K**				**I**	**L**	**S**	**S**
Kim et al. (2007)																										
Gp I	1	3														V		**H**								
Gp IIa	34	10							*	*	S		*	E*					**K**							
Gp IIb	21	3						I								**V**			**K**							
Gp IIc	11	2														**T**			**K**							
Gp I+IIb		1						**I**								**V**		**H**	**K**							
Garcia-Cobos et al. (2007)																										
Gp I	5	5	*	N	G*			I						E		V		**H**				S				
Gp IIa	20			N				*				A		E					**K**		S					
Gp IIb	42	11		N				I			S			E		**V**			**K**							
Gp IIc	98	14		N						*	S					**T**			**K**							
Gp IId	9	3											**V**			T			**K**							
Gp III-like	12			**N**		**N**		**I**	**T**	F								**H**				**S**				
Skaare et al. (2009)																										
Gp II-	1			**N**										**E**					**K**		**S**					
Gp IIa	1																		**K**							
Gp IIb	13			**N**				**I**						E		**V**			**K**				**I**		**S**	
Gp IIc	1															**T**			**K**							
Gp IId	7												**V**						**K**				**I**		**S**	

[a] Boldface type, all isolates; underline, most isolates; plain text, some isolates; *, amino acid substitution identified at adjacent position.

Strain	bla (-)	bla (+)	Amino acid at position[a]: 337	350	352	357	368	377	385	389	437	443	449	490	501	502	511	517	526	528	530	532	547	562	569	586
Rd			**A**	**D**	**S**	**S**	**A**	**M**	**S**	**L**	**A**	**T**	**I**	**G**	**R**	**A**	**V**	**R**	**N**	**Y**	**T**	**T**	**V**	**V**	**N**	**A**
Shuel and Tsang (2009)																										
Gp IIa	1													**E**					**K**		**S**					
Gp IIb	14	2		N				I						E		**V**			**K**				I		S	
Gp IId	4		*		G	*		I					**V**						**K**				I		**S**	
Bae et al. (2010)																										
Gp I	3	2		**N**		**D**		**I**	**T**									**H**								
Gp IIa	3	2		N				I	T					E					**K**							
Gp IIb	10	17		N				I						E		**V**			**K**							
Gp IIc	10	4		N		D	T	I	T	F	S		V			**T**			**K**							
Gp IId	3	2											**V**						**K**							
Gp III	3			**N**		**D**		**I**	**T**	**F**									**K**							
Barbosa et al. (2011)																										
Gp I	3																	**H**					*			
Gp IIa	7	1		N				I						E					**K**		S		I*			
Gp IIb	55	40	*	N		N		I			S	*		E		**V**	A		**K**	*			I*		S	
Gp IIc	17	6		N				I								**T**			**K**				I		S	
Gp IId	10			N							S		**V**			T			**K**				**I**		**S**	
Gp III-like	2			**N**		**N**		**I**	**T**									**H**				**S**	**I**			
Park et al. (2013)																										
Gp I	1			**N**														**H**					**I**		**S**	
Gp IIa	4						T												**K**		S		**I**		S	
Gp IIb	15	17		N				I								**V**			**K**				**I**		S	
Gp IIc	1	1		N		N	T									**T**			**K**							
Gp IId	8	2											**V**						**K**				I		S	
Gp III	16	7		**N**		**N**		**I**	**T**	**F**									**K**				I	**L**	S	
Gp III+IIc	2	1		**N**		**N**		**I**	**T**	**F**						**T**			**K**							
Gp III-like	2	1		**N**		**N**		**I**	**T**									**H**				**S**	**I**			

[a] Boldface type, all isolates; underline, most isolates; plain text, some isolates; *, amino acid substitution identified at adjacent position.

Similar to the earlier study of Ubukata et al. (2001) additional miscellaneous substitutions at positions 350, 357, 377, and 437 were also frequently identified in some, but not all of the French isolates (Dabernat et al., 2002).

In a study of European BLNAR and BLPACR isolates identified by an amoxicillin MIC of ≥ 2 µg/mL, or an amoxicillin-clavulanate MIC of ≥ 8 µg/mL (for BLPACR isolates), no Gp III BLNARs were identified on *ftsI* gene characterisation (Dabernat et al., 2002; Fluit et al., 2005). Instead, the majority of BLNAR isolates (21/26) had the Gp IIb and Gp IIc substitution profiles. Interestingly, two isolates defined as BLNAR by phenotype exhibited no substitutions in PBP3 when compared to the Rd reference sequence, but unfortunately these isolates did not undergo further characterisation (Fluit et al., 2005).

More recently, separate studies characterizing PBP3 in Portuguese, Norwegian and Spanish clinical isolates identified that the Gp IIb and Gp IIc substitution profiles still accounted for the majority of BLNAR isolates identified throughout Europe (Garcia-Cobos et al., 2007; Skaare et al., 2010; Barbosa et al., 2011). Similar to the findings of Fluit et al. (2005), neither study detected any Gp III BLNAR isolates, although Garcia-Cobos et al. (2007) identified 12 isolates (5%) that contained the Gp III amino acid triplet near the SSN motif (M377I, S385T, and L389F) in conjunction with R517H instead of the previously reported N526K substitution (Fluit et al., 2005; Garcia-Cobos et al., 2007). As a result these isolates were termed Gp III-like (Garcia-Cobos et al., 2007). Similar to other studies, miscellaneous substitutions at positions 350, 437, 490, 530, 547 and 569 were also frequently observed among the N526K-

positive European isolates (Fluit et al., 2005; Garcia-Cobos et al., 2007; Skaare et al., 2010; Barbosa et al., 2011).

In a study of 621 Japanese *H. influenzae* isolates cultured from patients with CAP a single isolate showed a new deduced amino acid substitution at position 526 of PBP3 (Sanbongi et al., 2006). In this isolate asparagine was substituted for histidine at position 526 (N526H) instead of the lysine commonly seen in BLNAR (Sanbongi et al., 2006). This is the first report of the N526H substitution in *H. influenzae* and unfortunately, the resultant effect on ampicillin MIC was obscured by the presence of a TEM-1 β-lactamase (Sanbongi et al., 2006). As the N526H substitution has most recently been described as an important BLNAR-defining substitution in isolates of *Haemophilus parainfluenzae*, the importance of this substitution in *H. influenzae* still needs to be clarified (Garcia-Cobos et al., 2013).

The genetic characterisation of PBP3 in BLNAR and BLPACR isolates from Korea revealed similar prevalence's to those in Europe, with the Gp IIb, Gp IIc and Gp IId substitutions profiles occurring most frequently (Kim et al., 2007; Bae et al., 2010; Park et al., 2013). Interestingly, high-BLNAR isolates with the Gp III and Gp III-like genotypes were only recently recognised in Korea (Park et al., 2013). Similar to other studies additional substitutions at positions 350, 357, 368, 532, 547, 562 and 569 were frequently observed (Kim et al., 2007; Bae et al., 2010; Park et al., 2013).

It is difficult to compare the PBP3 profiles of the BLNAR and BLPACR isolates studied by Straker et al. (2003), as they were pre-selected on the basis of cefuroxime resistance (MIC ≥ 2 μg/mL) and not ampicillin (Straker et al., 2003). In the 14 cefuroxime resistant isolates studied several commonly reported BLNAR

substitutions were identified, including; D350N, S357N, M377I, A502T/V, R517H and N526K (Straker et al., 2003). Interestingly, previously unreported substitutions of A337V, T443A, R501L, V511A, Y528H and T532S were described among the cefuroxime resistant isolates of that study. However, some of these substitutions particularly those at positions 443, 511 and 532 have been infrequently reported in some more recent BLNAR studies, although no link has been made between the presence of these substitutions and cefuroxime resistance (Kubota et al., 2006; Garcia-Cobos et al., 2007; Barbosa et al., 2011).

2.3.4 BLNAR Substitutions and their Correlation with MIC

On the basis of transformational studies, the presence of either the R517H or N526K substitution results in a two-to-four fold increase in ampicillin MIC, and a two-to-eight fold increase in MIC to most cephalosporin's, although the absolute degree of resistance or reduced susceptibility is dependent on the particular substitutions present (Ubukata et al., 2001; Osaki et al., 2005; Sanbongi et al., 2006). Two studies have evaluated the effect that M377I, S385T, L389F, R517H and N526K have on susceptibility to β-lactams, either through transformation of Rd with *ftsI* genes from clinical BLNAR isolates, or through the introduction of individual substitutions into the Rd backbone by site-direct mutagenesis (Ubukata et al., 2001; Osaki et al., 2005; Sanbongi et al., 2006). Irrespective of the method used the results are similar, such that the introduction of the R517H substitution into Rd results in a two-fold and four-fold increase in ampicillin (1 μg/mL) and cefotaxime MICs (0.063 μg/mL), respectively, compared to the non-susceptible baseline (AMP

MIC 0.25 μg/mL; CTX MIC 0.008 μg/mL) (Ubukata et al., 2001). Subsequent studies established that the step-wise addition of the Gp III substitutions M377I, S385T and L389F, to a R517H backbone further increased the MICs to ampicillin and various cephalosporin's whilst, minimal changes were noted in the imipenem and meropenem MICs of the resultant transformants (Osaki et al., 2005; Sanbongi et al., 2006).

Likewise, transformation of *H. influenzae* Rd with the *ftsI* gene from an N526K-positive donor resulted in a significant increase in MIC (two- to eight-fold) for aminopenicillins and all cephalosporin's (except ceftazidime), respectively compared to the baseline (Osaki et al., 2005). Similar to what is seen with the R517H substitution, addition of substitutions at positions 377, 385, and 389 into the N526K backbone, further increased MICs to most β-lactam antibiotics (especially the cephalosporin's), but not meropenem (Osaki et al., 2005). Interestingly, both studies concluded that the addition of the M377I substitution alone to either the R517H or N526K-positive backbone had no effect on β-lactam resistance (Osaki et al., 2005; Sanbongi et al., 2006). This suggests that the M377I substitution may be a coincidental artefact of the S385T and L389F substitutions (Osaki et al., 2005; Sanbongi et al., 2006).

Straker et al. (2003) evaluated the contribution of *ftsI* gene mutations on cefuroxime resistance specifically and found that deduced PBP3 substitutions S357N, A502T/V, R517H and N526K were common among cefuroxime resistant isolates (MIC ≥ 8 μg/mL) (Straker et al., 2003). However, using transformation and protein modelling experiments, the authors of that study concluded that the S357N,

R501H and V511A substitutions in conjunction with R517H were most likely responsible for cefuroxime resistance (Straker et al., 2003).

2.4 Defining, Detecting and Screening for Isolates with Altered PBP3s

Currently, there is no consensus on how to define BLNAR isolates. Some investigators define BLNAR isolates phenotypically by antimicrobial susceptibility testing (AST), however, this approach is significantly influenced by the susceptibility breakpoints imposed by AST regulatory bodies and the testing methodology adopted (Garcia-Cobos et al., 2008b). Using phenotypic descriptors, BLNAR isolates are defined as any β-lactamase-negative isolate that exhibits non-susceptibility (≥ 2 μg/mL) or resistance (≥ 4 μg/mL) to ampicillin (CLSI, 2013). Conversely, other investigators advocate that detection of the resistance mechanism should be used to define BLNAR isolates. In this instance BLNAR isolates are strictly defined as any β-lactamase-negative isolate harbouring either an R517H or N526K substitution in PBP3, even though it is now routinely recognised that the presence of the resistance mechanism does not always correlate with phenotypic resistance to ampicillin (Tristram et al., 2007).

The ambiguity surrounding how to define BLNAR isolates significantly influences ones ability to reliably detect them in the diagnostic setting. For example detection of BLNAR isolates is dependent on; the BLNAR definition used, the phenotypic or genotypic methodology used for detection (MIC or disc diffusion versus SNP PCR or *ftsI* gene sequencing), and the correlation between phenotypic resistance and the

presence of the genotypic resistant mechanism. These inter-related factors are further complicated when one also considers the detection of BLPACR isolates.

In an attempt to simplify the current approaches used by authors to screen for and identify isolates with altered PBP3s (irrespective of β-lactamase status), an overview of the commonly used phenotypic and genotypic approaches to define and detect BLNAR and BLPACR isolates follows.

2.4.1 Defining BLNAR and BLPACR Isolates

2.4.1.1 Phenotypic Definitions

The susceptibility of *H. influenzae* isolates to commonly prescribed β-lactam antibiotics is currently inferred by an isolates intrinsic susceptibility to ampicillin (CLSI, 2013). Using current CLSI guidelines, susceptibility to ampicillin in *H. influenzae* is determined by the MIC breakpoints of; susceptible ≤ 1 μg/mL; intermediate 2 μg/mL and resistant ≥ 4 μg/mL (CLSI, 2013). According to these breakpoints, a BLNAR isolate is strictly defined as any β-lactamase-negative isolate that has an ampicillin MIC above the resistant breakpoint of ≥ 4 μg/mL (CLSI, 2013). However, as isolates with ampicillin MICs of 2 μg/mL are interpreted as ampicillin non-susceptible, some authors also include these intermediate isolates in their BLNAR definitions (Tristram et al., 2007). The term β-lactamase-negative ampicillin-intermediate (BLNAI) has been used to describe these isolates with intermediate susceptibilities, although this terminology has not yet been widely adopted (Niki et al., 2011).

The phenotypic definition of BLNAR isolates is further confused by the different recommendations made by different AST regulatory bodies; British Society for Antimicrobial Chemotherapy (BSAC), European Committee on Antimicrobial Susceptibility Testing (EUCAST), and CLSI. According to the current AST breakpoints from these agencies (summarised in Table 2.3), BLNAR isolates can be defined as any β-lactamase-negative isolate with an ampicillin MIC of ≥ 2 μg/mL according to BSAC, or ≥ 4 μg/mL according to both CLSI and EUCAST, respectively (Andrews, 2009; EUCAST, 2013; CLSI, 2013).

Unfortunately, the molecular characterisation of BLNAR isolates has identified that the majority of BLNAR isolates have ampicillin MICs in the range of 0.5 to 2 μg/mL and this further complicates matters as they significantly overlap with those of the ampicillin susceptible and intermediate populations currently defined by all microbiological susceptibility breakpoints (Figure 2.1).

In addition, the phenotypic definition of BLPACR isolates is confused by similar factors. According to CLSI susceptibility breakpoints, BLPACR isolates are defined as any β-lactamase-positive isolate that is resistant to amoxicillin-clavulanate (≥ 8/4 μg/mL) (CLSI, 2013). However, both EUCAST and BSAC use a breakpoint of > 2 μg/mL to define resistance to amoxicillin-clavulanate (EUCAST, 2013; CLSI, 2013). Similar to what has been shown in BLNAR isolates, the genotypic characterisation of BLPACR isolates has identified that the many BLPACR isolates are actually susceptible to or only have reduced susceptibility to amoxicillin-clavulanate, and as a result would go undetected (Figure 2.2).

Table 2.3 Ampicillin susceptibility breakpoints commonly used to define and detect BLNAR and BLPACR isolates of *H. influenzae,* by the MIC and disc diffusion methodologies.

Regulatory Body[b]	Interpretative Criteria/Susceptibility Breakpoints[a]							BLNAR/BLPACR Definition
	MIC (µg/mL)			Disc Diffusion (mm)				
	S	I	R	Disc Potency	S	I	R	
Ampicillin								
CLSI	≤ 1	2	≥ 4	10 µg	≥ 22	19-21	≤ 18	BLN with ampicillin MIC ≥ 4 µg/mL
EUCAST*	≤ 1	-	≥ 2	2 µg	≥ 16	-	< 16	BLN with ampicillin MIC ≥ 2 µg/mL
BSAC	≤ 1	-	≥ 2	2 µg	≥ 18	-	≤ 17	BLN with ampicillin MIC ≥ 2 µg/mL
Amoxicillin-clavulanate**								
CLSI	≤ 4	-	≥ 8	20/10 µg	≥ 20	-	≤ 19	BLP with ampicillin MIC ≥ 8 µg/mL
EUCAST	≤ 2	-	> 2	2/1 µg	≥ 14	-	≤ 13	BLP with ampicillin MIC > 2 µg/mL
BSAC	≤ 2	-	> 2	2/1 µg	≥ 15	-	< 15	BLP with ampicillin MIC > 2 µg/mL

[a] S, Susceptible; I, Intermediate; R, Resistant.

[b] CLSI, Clinical Laboratory and Standards Institute; EUCAST, European Committee on Antimicrobial Susceptibility Testing; BSAC, British Society for Antimicrobial Chemotherapy.

[*] In early 2013 EUCAST released new recommendations for the detection of BLNAR isolates, where a 1-unit benzylpenicillin screen and a test for β-lactamase production is to be used for the separation of fully susceptible isolates from those with β-lactamase mediated resistance (BLPAR) and those with altered PBP3 mediated resistance (BLNAR) (EUCAST, 2013).

[**] Concentrations of amoxicillin to clavulanate are 2:1.

This highlights the fact that current microbiologically based breakpoints are problematic for the definition and detection of BLNAR and BLPACR isolates, as these isolates do not always express clinical resistance (Tristram et al., 2007). In response to the overlapping nature of the ampicillin and amoxicillin-clavulanate MICs of BLNAR and BLPACR isolates, some authors now subcategorise BLNAR isolates according to the magnitude of their MIC increase into: low-BLNAR (AMP MIC 0.5 - 2 μg/mL), high-BLNAR (AMP MIC ≥ 4 μg/mL), low-BLPACR (also termed BLPACR-I; AMP MIC ≥ 64 μg/mL) and high-BLPACR (also termed BLPACR-II; AMP MIC ≥ 64 μg/mL). Although these definitions give a more specific explanation of the resistance profile of an isolate, they further confuse current approaches used in phenotypic detection.

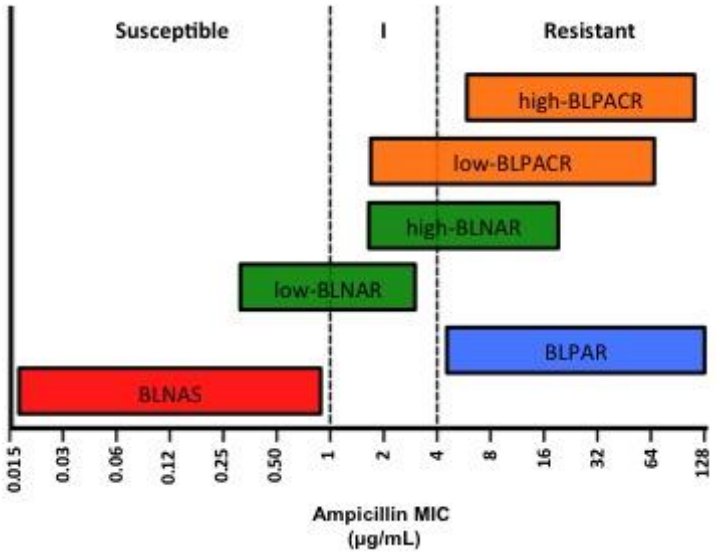


Figure 2.1 Overlapping nature of ampicillin MICs for β-lactam resistance classes in isolates of *H. influenzae* according to CLSI susceptibility breakpoints.

Susceptible and resistant denotes the current CLSI susceptibility breakpoints for ampicillin. Susceptible ≤ 1 µg/mL, Intermediate 2 µg/mL, Resistant ≥ 4 µg/mL.

Data adapted from Hasegawa et al. (2004), Hasegawa et al. (2006), Garcia-Cobos et al. (2007), Kim et al. (2007), and Barbosa et al. (2011).

BLNAS, β-lactamase-negative ampicillin-sensitive; BLPAR, β-lactamase-positive ampicillin-resistant; low-BLNAR, β-lactamase-negative ampicillin-resistant isolates with low ampicillin MICs (0.5 - 4 µg/mL) and the Gp I or Gp II genotype; high-BLNAR, β-lactamase-negative ampicillin-resistant isolates with high ampicillin MICs (4 - 16 µg/mL) and the Gp III genotype; low-BLPACR, β-lactamase-positive amoxicillin-clavulanate-resistant isolates with the Gp I or Gp II genotype; high-BLPACR, β-lactamase-positive amoxicillin-clavulanate-resistant isolates Gp III genotype.

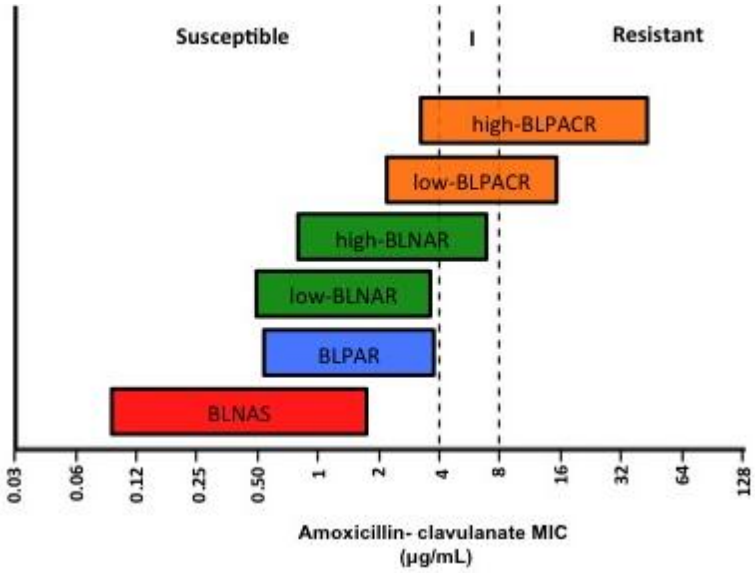


Figure 2.2 Overlapping nature of amoxicillin-clavulanate MICs for β-lactam resistance classes in isolates of *H. influenzae* according to CLSI susceptibility breakpoints.

Susceptible and resistant denotes the current CLSI susceptibility breakpoints for amoxicillin-clavulanate. Susceptible ≤ 4 µg/mL, and Resistant ≥ 8 µg/mL.

Data adapted from Hasegawa et al. (2004,) Hasegawa et al. (2006), Garcia-Cobos et al. (2007), Kim et al. (2007), and Barbosa et al. (2011).

BLNAS, β-lactamase-negative ampicillin-sensitive; BLPAR, β-lactamase-positive ampicillin-resistant; low-BLNAR, β-lactamase-negative ampicillin-resistant isolates with low ampicillin MICs (0.5 - 4 µg/mL) and the Gp I or Gp II genotype; high-BLNAR, β-lactamase-negative ampicillin-resistant isolates with high ampicillin MICs (4 - 16 µg/mL) and the Gp III genotype; low-BLPACR, β-lactamase-positive amoxicillin-clavulanate-resistant isolates with the Gp I or Gp II genotype; high-BLPACR, β-lactamase-positive amoxicillin-clavulanate-resistant isolates Gp III genotype.

2.4.1.2 Genotypic Definitions

In the strictest sense the presence of either the R517H or N526K substitution in PBP3 defines the genotype of a BLNAR or BLPACR isolate. This genotypic definition is currently the gold standard for BLNAR and BLPACR identification, as it can be conveniently applied to any *H. influenzae* isolate irrespective of β-lactamase status and phenotypic susceptibility profile.

When a BLNAR or BLPACR isolate has been identified by genotype it is generally termed genotypically BLNAR (gBLNAR), however some authors further characterise the genotype of BLNAR and BLPACR isolates by the presence of additional substitutions in PBP3 as well as the methodology used for genotypic detection. For example, some authors use direct sequencing of the *ftsI* gene, and categorise BLNAR/BLPACR isolates into genotypic groups on the basis of key BLNAR-defining and BLNAR-associated substitutions in PBP3 (Ubukata et al., 2001; Dabernat et al., 2002). Other authors indirectly screen for the PBP3 substitutions N526K and S385T by PCR, and use this to infer a BLNAR genotype (Hasegawa et al., 2003; Nakamura et al., 2009). As a result there are currently a lot of interrelated terms used by authors to define the genotype of an isolate with altered PBP3s, and these are summarised in Table 2.4. (Ubukata et al., 2001; Dabernat et al., 2002; Garcia-Cobos et al., 2007).

Table 2.4 Genotypic classification systems and commonly used nomenclature for defining BLNAR and BLPACR isolates by genotype.

Method	Classification	Genotypic Requirements with respect to PBP3[a]	Other	Reference
ftsI sequence				
	Gp I	R517H	-	Ubukata et al. (2001)
	Gp II	N526K	-	Ubukata et al. (2001)
	Gp IIa	N526K	-	Dabernat et al. (2002)
	Gp IIb	N526K and A502V	-	Dabernat et al. (2002)
	Gp IIc	N526K and A502T	-	Dabernat et al. (2002)
	Gp IId	N526K and I449V	-	Dabernat et al. (2002)
	Gp III	N526K, M377I, S385T, L389F	-	Ubukata et al. (2001)
	Gp III-like	R517H, M377I, S385T, L389F	T532S, D350N, S357N	Garcia-Cobos et al. (2007)
PCR Classification				
	glow-BLNAR	N526K only		Hasegawa et al. (2003)
	ghigh-BLNAR	N526K and S385T		Hasegawa et al. (2003)
	gBLPACR I	N526K only (low-BLNAR)	BLP +	Hasegawa et al. (2004)
	gBLPACR II	N526K and S385T (high-BLNAR)	BLP +	Hasegawa et al. (2004)
General Classification				
	BLNAI	N526K or R517H	Ampicillin MIC ≥ 2 µg/mL	Farrell et al. (2005)
	Low-BLNAR	Gp I or Gp II genotype	Ampicillin MIC 0.5 – 4 µg/mL	Hasegawa et al. (2003)
	High-BLNAR/BLNAR	Gp III or Gp III-like genotype	Ampicillin MIC ≥ 4 µg/mL	Hasegawa et al. (2003)
	gBLNAR	N526K or R517H	-	Hotomi et al. (2007)

[a] Isolates must have at least these substitutions to be assigned to the corresponding group; however, other additional substitutions may be present.

2.4.2 Detecting BLNAR and BLPACR Isolates By Phenotype

2.4.2.1 Using MIC

Detecting BLNAR isolates phenotypically by MIC is problematic. Problems arise through a lack of consensus on how to define BLNAR isolates phenotypically, the extreme variability in ampicillin MIC exhibited by BLNAR isolates, and the lack of correlation between ampicillin MIC and clinical resistance. According to current CLSI recommendations, BLNAR isolates are defined by an ampicillin MIC of ≥ 4 μg/mL, even though it is universally accepted that the majority of BLNAR isolates actually have ampicillin MICs in the range of 0.5 to 2 μg/mL. This lack of correlation between ampicillin MIC, clinical resistance and AST breakpoints makes the detection of BLNAR isolates by current susceptibility breakpoints unreliable (Shown in Figure 2.1). In an attempt to overcome this limitation numerous authors have modified these ampicillin breakpoints to include ampicillin MICs of ≥ 0.5 μg/mL, ≥ 1 μg/mL, ≥ 2 μg/mL when screening for BLNAR isolates in surveillance studies (Doern et al., 1997; Ubukata et al., 2001; Dabernat et al., 2002; Barbosa et al., 2011).

The ability to reliably detect BLNAR isolates by MIC is further affected by the inherent methodological issues in microbroth dilution testing. There is a natural variation of ± one doubling dilution in MIC testing, meaning that isolates with ampicillin MICs that cluster around the susceptibility breakpoints could be classified as either susceptible, intermediate or resistant on repeat testing (Figure 2.1) (Scriver et al., 1994a; Barry et al., 2001; Tristram et al., 2007).

Additional issues with MIC testing for BLNAR detection stem from the fact that BLNAR isolates exhibit reduced susceptibility to other β-lactam antibiotics, and that

PBP3 has the highest binding affinity for cephalosporin's (Hasegawa et al., 2003; Hasegawa et al., 2004; Hasegawa et al., 2006). Consequently, some authors have investigated the use of alternative β-lactam antibiotics to screen for BLNAR isolates in surveillance studies with varying success (James et al., 1996; Straker et al., 2003; Fluit et al., 2005; Garcia-Cobos et al., 2008b). For example, Fluit et al. (2005) and Garcia-Cobos et al. (2008a) used amoxicillin MICs of ≥ 2 μg/mL and ≥ 0.5 μg/mL to detect and define BLNAR isolates in two European studies respectively (Fluit et al., 2005; Garcia-Cobos et al., 2008b). Whilst other authors have used cephalosporin's such as cefaclor, cefotaxime or cefuroxime for BLNAR detection, making it difficult to directly compare their findings to those where ampicillin resistance is used as the marker to define and detect BLNAR isolates (James et al., 1996; Livermore and Brown, 2001; Straker et al., 2003).

2.4.2.2 Using Disc Diffusion

Disc diffusion screening for BLNAR isolates introduces another layer of uncertainty to BLNAR detection. Here issues stem from the fact that disc diffusion testing is based on the premise that the zone of inhibition is proportional to the MIC, although in reality this is not always the case (Turnidge and Paterson, 2007). Methodological differences such as choice of testing medium, antibiotic type and antibiotic disc strength (high-strength versus low-strength discs) further affect the ability of the disc diffusion assay to reliably detect BLNAR isolates (Zerva et al., 1996; Karpanoja et al., 2004; Garcia-Cobos et al., 2008b). Of particular concern is the choice of antibiotic disc strength, as this significantly influences the degree of

correlation between zone size (mm), and MIC (Turnidge and Paterson, 2007; Garcia-Cobos et al., 2008b).

Historically, disc diffusion methodologies used for the detection of β-lactam resistance in *H. influenzae*, have used high-strength antibiotic discs, however recent evaluations have reported a poor correlation between zone diameter and detection of BLNAR mediated resistance with these high-strength discs (Mendelman et al., 1986; Heelan et al., 1992; Scriver et al., 1994b; Zerva et al., 1996; Karpanoja et al., 2004). For example, use of the 10 μg ampicillin disc currently recommended by CLSI, has been shown to incorrectly categorise 81.3% of BLNAR isolates as either ampicillin intermediate or susceptible when CLSI susceptibility criteria are applied (Susceptible ≥ 22 mm; Intermediate 19-21 mm; Resistant ≤ 18 mm; calibrated to MICs of ≤ 1 μg/mL, 2 μg/mL and ≥ 4 μg/mL, respectively) (Zerva et al., 1996). Similar discrepancies have been reported by other authors using the 10 μg ampicillin disc for BLNAR detection, and in these studies attributed the poor performance of the assay to the highly overlapping nature of the zone diameters of both the BLNAS and BLNAR populations (Karpanoja et al., 2004; Garcia-Cobos et al., 2008b).

Recent attempts to improve the ability of the ampicillin disc diffusion test to separate BLNAR and BLNAS populations has involved the use of low-strength ampicillin discs (2 μg), or the modification of interpretative zone sizes (Zerva et al., 1996; Karpanoja et al., 2004). Evaluations of the low-potency 2 μg ampicillin disc [using breakpoints of: Susceptible ≥ 17 mm; Resistant ≤ 13 mm proposed by Zerva et al. (1996)], was shown to significantly improved the sensitivity and specificity of the assay for BLNAR detection (92% and 90% versus 71% and 88% for the 10 μg

disc, respectively), even though the breakpoints are calibrated to the same ampicillin MICs (Karpanoja et al., 2004). In contrast, a study by Garcia-Cobos et al. (2008a) found an overall poor correlation between disc diffusion testing methodologies (using both high- and low-strength discs) and BLNAR detection (Garcia-Cobos et al., 2008b). Admittedly in this study, Garcia-Cobos and colleagues (2008b) improved the study design, by using molecularly characterised gBLNAS and gBLNAR populations, compared to the phenotypically defined populations used in the aforementioned studies (Zerva et al., 1996; Karpanoja et al., 2004; Garcia-Cobos et al., 2008b).

More recently Norskov-Lauritsen et al. (2011) compared the performance of the CLSI recommended ampicillin (10 µg), cefaclor (30 µg) and cefuroxime (30 µg) discs for the detection of low-BLNAR isolates. Although they did not encounter the detection inconsistencies noted by previous authors with respect to the 10 µg ampicillin disc, they found the cefaclor 30 µg disc using major zone breakpoints of 23/22 mm on chocolate agar to be the most sensitive (98%) and specific (99%) for BLNAR detection (Norskov-Lauritsen et al., 2011). The authors of this study used the designation of an isolate as BLNAR by *ftsI* gene sequence as the gold-standard, (rather than ampicillin MIC), which is an improvement in study design, although, the fact they pre-selected isolates for *ftsI* sequencing by cefaclor zone sizes (≤ 24 mm on chocolate agar, ≤ 28 mm on ST agar) makes it difficult to fully interpret their conclusions (Norskov-Lauritsen et al., 2011).

Despite the lack of correlation between disc strength, zone size and MIC seen in disc diffusion testing, only EUCAST and BSAC have currently adopted the use of the 2 µg

ampicillin disc for BLNAR detection (Andrews, 2009; EUCAST, 2013). In fact, in the revised interpretative standards from EUCAST (2013), a 1 unit benzylpenicillin screen using a resistant cut-off of < 12 mm has replaced the ampicillin 2 µg disc for the detection of β-lactam resistance in *H. influenzae* (EUCAST, 2013). With this screen any β-lactamase-negative isolate with zones of < 12 mm to benzylpenicillin are recognised as BLNAR, and the success of this approach was recently highlighted by Sondergaard and colleagues (2012) (Sondergaard et al., 2012; EUCAST, 2013).

It is important to note that the inconsistencies surrounding the ability to detect BLNAR isolates phenotypically has developed from the fact that the current susceptibility breakpoints used for BLNAR detection, were originally designed to separate the BLPAR and BLNAS populations. Until the susceptibility breakpoints are revised for the specific detection of BLNAR and BLPACR populations, disagreements between MIC breakpoints, disc diffusion zones, the ability to correctly differentiate BLNAR and BLPACR populations from other phenotypic populations, will exist.

2.4.3 Detecting BLNAR and BLPACR Isolates By Genotype

Problems with MIC based methodologies and susceptibility breakpoints have prompted a move toward genotypic detection of BLNAR and BLPACR isolates. Common genotypic approaches include sequencing of the *ftsI* gene or single nucleotide polymorphism (SNP)-based PCR targeting the important BLNAR-defining substitutions of PBP3.

2.4.3.1 Using *ftsI* gene Sequencing

Sequencing of the *ftsI* gene is the gold standard for BLNAR detection as it is the only method by which both BLNAR-defining substitutions (R517H and N526K), as well as other additional PBP3 substitutions, can be detected (Ubukata et al., 2001). Although the most frequently used approach to *ftsI* gene sequencing only targets the transpeptidase domain of PBP3, some authors sequence the entire *ftsI* gene to collect additional genotypic data (Osaki et al., 2005).

Not only does *ftsI* gene sequencing allow for the characterisation of *ftsI* gene mutations and the encoded substitutions in PBP3, it also allows for the detection of BLNAR isolates with low ampicillin MICs that may be missed by other phenotypic detection methods. However, as gene sequencing requires specialised and expensive expertise, it is not a viable option for all laboratories. As a result numerous studies apply phenotypic screens (such as an AMP MIC ≥ 1 µg/mL or ≥ 2 µg/mL) to large surveillance data sets to pre-select isolates for subsequent *ftsI* sequencing (Barbosa et al., 2011).

2.4.3.2 Using SNP-Based PCR Assays

To standardise the method of BLNAR detection in surveillance studies, Hasegawa et al. (2003) developed a simple PCR based algorithm for the detection of all β-lactam resistance mechanisms in *H. influenzae* (Hasegawa et al., 2003). This PCR algorithm relies on the amplification of conserved regions of five important *Haemophilus* genes; the *omp P6* (or as more recently recommended, the 16S rRNA gene) for *H.*

influenzae identification; the *capB* gene encoding the Hib capsule; the bla_{TEM} and bla_{ROB} genes encoding β-lactamase production; and finally two PCRs targeting a normal (susceptible) and abnormal (resistant) fragment of the *ftsI* gene for detection of non-β-lactamase-mediated ampicillin-resistance (Hasegawa et al., 2003). As a result, isolates are identified as either NTHi or HIb, and can be conveniently classified into one of six "susceptibility" genotypes: gBLNAS, no resistance mechanism detected; gBLPAR, only β-lactamase resistance genes detected; glow-BLNAR, only the N526K substitution detected; gBLNAR (also termed ghigh-BLNAR by some authors) both the N526K and S385T substitutions detected; gBLPACR-I, β-lactamase genes and the N526K substitution detected; gBLPACR-II, β-lactamase genes and the N526K and S385T substitutions detected (Hasegawa et al., 2003; Hasegawa et al., 2006).

In this approach, there are two PCR assays designed to amplify different regions of the *ftsI* gene for BLNAR detection. One assay targets the AAT codon at position 1576-1578 of the *ftsI* gene that encodes the normal N526 of BLNAS isolates (assay termed PBP3-S), whilst the other targets two separate codon changes: AGT to ACT codon change at position 1153-1155 of the *ftsI* gene encoding S385T of high-BLNAR isolates, and an AAT to AAG change at position 1576-1578 encoding the BLNAR-defining N526K substitution (Hasegawa et al., 2003). Although these SNP-based PCR assays specifically target and amplify N526 of normal BLNAS isolates (PBP3-S assay), and N526K and S385T of high-BLNAR isolates (PBP3-BLN assay), neither assay positively detects through amplification the most common BLNAR genotype of N526K only (Hasegawa et al., 2003). In contrast, with these assays the low-BLNAR genotype (N526K only) is detected when no-amplification is observed with either

PCR reaction and this poses some methodological issues with reliability (Hasegawa et al., 2003). In an attempt to rectify the designation of low-BLNAR through no-amplification, Nakamura et al. (2009) developed a modified PCR assay that amplified in the presence of the low-BLNAR substitution N526K, and in the presence of N526K and S385T, although this methodology is yet to be widely adopted (Nakamura et al., 2009).

More recently, Kishii et al. (2011) developed a real-time PCR assay that used cyclic probes to simultaneously detect both the N526K and S385T substitutions in PBP3 independently of each other (Kishii et al., 2011). Although this assay is more sensitive and specific for BLNAR detection than the conventional gel-based PCR assay of Hasegawa et al. (2003), it failed to detect N526K and S385T in a number of gBLNAR isolates (10/206), due to a one bp discrepancy in the respective probe regions (Kishii et al., 2011). As a result the SNP-based PCR algorithm of Hasegawa et al. (2003) still remains the most widely adopted PCR assay for BLNAR surveillance and detection.

To date no SNP-based PCR assay has been designed to detect the other BLNAR-defining substitution R517H (Hasegawa et al., 2003). This is a significant limitation in methodology design, such that authors who use these PCR screens for BLNAR detection essentially bias the prevalence of N526K over R517H mediated resistance, due to the inability to detect R517H.

2.5 Epidemiology of BLNAR Isolates

The epidemiology of BLNAR mediated resistance in *H. influenzae* is complex. It is significantly influenced by a number of interrelated factors including; the natural competence of the *Haemophilus* species to uptake and incorporate genetic material from their surrounding environments into their genome and the diversification in population structure between NTHi and encapsulated isolates (Connor et al., 2012). In general, encapsulated isolates exhibit a highly clonal population structure, whilst NTHi isolates are considered to be much more phylogenetically diverse (Musser et al., 1986; Smith-Vaughan et al., 1998; Cerquetti et al., 2000; Connor et al., 2012). It is against this backdrop that many authors have attempted to elucidate the epidemiology and evolution of BLNAR mediated resistance in both serotypeable (particularly, Hib) and NTHi isolates of *H. influenzae*.

2.5.1 Independent Evolution of BLNAR

When BLNAR isolates were first described they were believed to have arisen by independent mutation in the *ftsI* gene and subsequently selected for by the pressures of antibiotic consumption (Ubukata et al., 2001). This was confirmed by early molecular characterisation studies, which showed a high level of sequence diversity in both the *ftsI* gene sequences and deduced amino acid sequences of PBP3 in BLNAR isolates (Dabernat et al., 2002; Barbosa et al., 2011). For example, analysis of PFGE and *ftsI* sequence data from the 108 BLNAR isolates studied by Dabernat et al. (2002) revealed a total of 70 different PFGE profiles, highlighting the heterogeneity of French BLNAR isolates (Dabernat et al., 2002). However, in the

same study there was also limited evidence to suggest the presence of a small number of BLNAR clones (Dabernat et al., 2002).

2.5.2 Clonal Spread of BLNAR

The dissemination and transmission of specific BLNAR clones has been reported by a number of authors to date (Karlowsky et al., 2002; Dabernat et al., 2002; Watanabe et al., 2004). In these reports, a small number of BLNAR isolates where shown to exhibit identical PFGE patterns and PBP3 substitutions profiles, and this was identified in both closed patient populations and hospital settings (Dabernat et al., 2002; Karlowsky et al., 2002; Watanabe et al., 2004).

More recently, the use of PFGE and *ftsI* gene sequencing in BLNAR characterisation studies has identified the presence of diverse *ftsI* gene sequences among clonally related BLNAR isolates, and identical *ftsI* gene sequences among genetically and geographically diverse NTHi isolates (Skaare et al., 2010; Barbosa et al., 2011). The documentation of identical *ftsI* gene sequences among genetically diverse BLNAR isolates, suggests that *ftsI* genes may be exchanged between isolates via horizontal gene transfer (Takahata et al., 2007; Skaare et al., 2010; Barbosa et al., 2011).

2.5.3 Horizontal Transfer of the *ftsI* gene

The ability of *H. influenzae* isolates to undergo horizontal transfer and recombination of the *ftsI* gene has recently been described (Takahata et al., 2007). During the routine analysis of the *ftsI* genes from 621 clinical *H. influenzae* isolates, Takahata and colleagues (2007) identified the presence of irregular mosaic *ftsI* gene

structures, where distinct sequence blocks were highly divergent to that of the reference sequence of *H. influenzae* Rd (Takahata et al., 2007). Subsequent analysis of the *ftsI* genes of other *Haemophilus* species identified a high degree of similarity between the divergent *ftsI* blocks and the *ftsI* sequence from the respiratory tract commensal *H. haemolyticus* (Takahata et al., 2007). Using *in vitro* transformation experiments, the authors successfully showed that isolates of *H. influenzae* could undergo horizontal transfer and recombination of the *ftsI* gene, and this resulted in the transmission of the BLNAR genotype (Takahata et al., 2007). Although the inter-species transfer of the *ftsI* gene was demonstrated in clinical isolates, no attempt was made in that study to confirm the inter-species recombination of the *ftsI* gene between *H. influenzae* and *H. haemolyticus in vitro*, or how recombination of the *ftsI* gene influenced the dissemination of the BLNAR genotype.

2.6 Identification and Separation of *H. influenzae* from *H. haemolyticus*

H. influenzae is most closely related to the respiratory commensal *H. haemolyticus*. Although *H. influenzae* and *H. haemolyticus* can be easily differentiated from other commensal *Haemophilus* species by their dependence on X and V co-factors for *in vitro* growth, their similar colony morphologies and biochemical activity make them difficult to separate phenotypically (Kilian, 2003; Murphy et al., 2007; Sandstedt et al., 2008). Historically, only the ability of *H. haemolyticus* to haemolyse horse blood agar could be used to separate the two species, however, with the subsequent recognition of non-haemolytic variants of *H. haemolyticus* the value of this phenotypic test is now limited (Murphy et al., 2007; Sandstedt et al., 2008).

Phylogenetically both *H. influenzae* and *H. haemolyticus* cluster closely together in the *Haemophilus sensu stricto* cluster on 16S ribosomal RNA (16S rRNA) analysis (Norskov-Lauritsen et al., 2005), although their type isolates form distinct phylogenetic branches allowing separation of the two species by further molecular analysis (McCrea et al., 2008; Kuhnert and Christensen, 2008). However in a more recent phylogenetic analysis of a collection of NTHi, haemolytic *H. haemolyticus*, and non-haemolytic *H. haemolyticus* isolates, a poor delineation or 'fuzzing' between the borders of these two species, was identified using the 16S rRNA technique (Norskov-Lauritsen et al., 2005; Norskov-Lauritsen, 2011). This highlights the current challenges laboratories face when attempting to separate non-haemolytic *H. haemolyticus* from typical NTHi isolates, particularly in respiratory tract specimens (McCrea et al., 2008; Norskov-Lauritsen et al., 2009; Binks et al., 2012).

2.6.1 Clinical Importance

As both *H. influenzae* and *H. haemolyticus* share the same environmental niche in humans they are both commonly encountered in the diagnostic laboratory from routine respiratory tract specimens. The ability to unambiguously differentiate between NTHi and *H. haemolyticus* (particularly non-haemolytic variants) is important diagnostically because only *H. influenzae* is currently considered pathogenic to humans, in a respiratory context (Murphy, 2003; Murphy et al., 2007). However, the retrospective analysis of *H. influenzae* culture collections, in recent years, has revealed that a high percentage of presumptively identified NTHi

are actually non-haemolytic *H. haemolyticus* following molecular analysis (Murphy et al., 2007). For example, in a retrospective analysis of 490 apparent NTHi isolates, it was identified that 39.5% (102/156) of the sputum isolates from COPD patients and 27.3% (12/32) of the NTHi isolates cultured from the nasopharynx of children, were actually non-haemolytic *H. haemolyticus* on molecular characterisation of the 16S rRNA gene (Murphy et al., 2007). Other studies have replicated these findings with 12% of presumptive NTHi from the nasopharynx of children identified as *H. haemolyticus* on the basis of 16S rRNA sequencing, whilst 16-21% of presumptive NTHi isolates from respiratory specimens were identified as non-haemolytic *H. haemolyticus* following further characterisation (Xie et al., 2006; Mukundan et al., 2007).

In contrast, Norskov-Lauritsen et al. (2009) found that only 0.4% (2/480) of presumptive NTHi isolates from clinical sources needed to be reclassified as *H. haemolyticus* on molecular characterisation, whilst Fenger et al. (2012) found only 0.5% (1/192) of NTHi from cystic fibrosis patients to be non-haemolytic *H. haemolyticus* on analysis of marker genes *fucK* and *sodC* (Norskov-Lauritsen, 2009; Fenger et al., 2012).

The mis-identification of *H. haemolyticus* as NTHi has been further compounded by recent studies that have isolated *H. haemolyticus* as the causative agent in a number of invasive infections (Jordan et al., 2011; Anderson et al., 2012). These findings highlight the possible pathogenic potential of *H. haemolyticus* isolates, an organism that until now has been strictly considered a bacterial commensal. This further emphasises the importance of being able to reliably separate *H.*

haemolyticus from the known pathogen *H. influenzae* in the diagnostic laboratory, and also highlights the current gap in the knowledge surrounding *H. haemolyticus*.

Of primary clinical concern is the high incidence of non-haemolytic *H. haemolyticus* mis-identified as NTHi from nasopharyngeal and oropharyngeal specimens (Hotomi et al., 2006; Murphy et al., 2007; Sandstedt et al., 2008), as such samples are frequently used for the surveillance of β-lactam resistance mechanisms in *H. influenzae*. In fact, many surveillance studies commonly use URT isolates of *H. influenzae* and inadequate methods for *H. influenzae* speciation, making it difficult to re-interpret the findings of these studies in light of *H. influenzae*/*H. haemolyticus* mis-identification (Murphy et al., 2007; Sandstedt et al., 2008; Chang et al., 2010). Furthermore, as findings from surveillance studies are routinely used to inform therapeutic guideline policies for both invasive and non-invasive diseases it is imperative that non-haemolytic *H. haemolyticus* be separated from NTHi to maximize the best possible therapeutic outcomes for the patient. Admittedly, as the current knowledge of β-lactam susceptibility in *H. haemolyticus* is limited, caution needs to be taken when interpreting susceptibility surveillance studies where isolates of *H. haemolyticus* might be inadvertently included.

2.6.2 Methods

Since the recognition of non-haemolytic isolates of *H. haemolyticus* the value of phenotypic methods for separation of NTHi from *H. haemolyticus* has significantly diminished (Kilian, 2003; Murphy et al., 2007; Sandstedt et al., 2008). Reliable separation instead requires molecular approaches that target either a single

conserved gene or multiple genetic targets conserved across the *H. influenzae* and *H. haemolyticus* genomes (Fung et al., 2006; Norskov-Lauritsen, 2009; Wang et al., 2011; Theodore et al., 2012; Binks et al., 2012).

A summary of the current molecular methods and their targets routinely used in the identification and separation of *H. influenzae* from *H. haemolyticus* is given in Table 2.5.

2.6.2.1 16S rRNA Sequencing

Sequencing of the highly conserved 16S rRNA gene is the current gold standard for the classification and identification of bacterial species (Norskov-Lauritsen, 2011). Phylogenetic analysis of 16S rRNA gene sequences from phenotypic *H. influenzae* isolates and type strains of closely related taxa (*H. haemolyticus* and *H. aegyptius*) has revealed two separate phylogenetic clusters of *H. influenzae* (phylogenetic group I and phylogenetic group II), that are separate to the distinct type cluster of *H. haemolyticus* (Norskov-Lauritsen et al., 2009). However, further phylogenetic tree analysis revealed that non-haemolytic isolates of *H. haemolyticus* formed a distinct variant cluster (also termed fuzzy or equivocal) that appears to be more closely related to *H. influenzae* than *H. haemolyticus* (Norskov-Lauritsen et al., 2009; Norskov-Lauritsen, 2009). Subsequent authors have confirmed the presence of this variant cluster on 16S rRNA analysis (Norskov-Lauritsen, 2011; Binks et al., 2012; Theodore et al., 2012).

Table 2.5 Current gene targets used for the identification and separation of *H. influenzae* from other closely related *Haemophilus* species.

Assay Details			Performance of Assay[c]	
Assay Type[a]	**Genetic Target[b]**	**Reference**	**Sensitivity (%)**	**Specificity (%)**
PCR:				
	fucK	Meats et al. (2003)	75.0	75.0
	igA	Vitovski et al. (2002)	88.9	91.7
	lgtC	McCrea et al. (2008)	80.6	94.8
	sodC	Kroll et al. (1995)	-	-
	omp P6	Vu et al. (2011)	94.4	62.5
	omp P2	Binks et al. (2012)	80.6	91.7
	16S rRNA	Murphy et al. (2007)	69.4	87.5
PCR/Probe:	*hap*	Norskov-Lauritsen et al. (2009)	-	-
	hpD	Wang et al. (2011)	88.9	91.7
	hpD	Wang et al. (2011)	88.9	91.7
Sequence/Phylogeny:	*recA*	Meats et al. (2003)	-	-
	16S rRNA	Murphy et al. (2007)	-	-

[a] PCR, polymerase chain reaction; PCR/Probe, polymerase chain reaction amplification of gene prior to specific probe use; HRM PCR, high resolution melt polymerase chain reaction; Sequence Phylogeny, sequencing of the gene target, prior to phylogenetic tree construction.

[b] *fucK*, fuculose kinase; *igA*, *Iga* protease; *lgtC*, lipo-oligosaccharide; omp P6, outer membrane protein P6; omp P2, outer membrane protein P2; *recA*, Rec A protein ; *sodC*, [Cu,Zn]-superoxide dismutase, *16S rRNA*, 16S rRNA; *hap*, *Haemophilus* adhesion and penetration protein; *hpD*, *Haemophilus* protein D.

[c] Assay performance data is from the Binks et al. (2011) studies, where most (but not all PCR assays) were evaluated for *H. influenzae* identification. In this study, the sensitivity and specificity of an assay was calculated using the designation of an isolates as *H. influenzae* or *H. haemolyticus* from concatenated 16S rRNA and *recA* gene phylogenies as the gold standard.

This suggests that although 16S rRNA sequencing is useful for the identification of most *H. influenzae* and *H. haemolyticus* isolates, the recent identification of intragenomic 16S heterogeneity in *Haemophilus* species, means that when used alone, 16S rRNA gene sequencing may no longer be sufficient for the delineation of these extremely closely related species (Norskov-Lauritsen, 2011; Binks et al., 2012).

2.6.2.2 MLST

Multi-locus sequencing typing (MLST) is a highly discriminating reference method used for the characterisation of bacterial isolates from different species (www.MLST.net). In 2003, the MLST scheme for *H. influenzae* was first described, and provided an unambiguous approach for characterising and comparing typeable and non-typeable isolates of *H. influenzae* worldwide (Meats et al., 2003). Although some authors argue that MLST is a more accurate approach for species designation as it uses sequence fragments (approximately 450-500 bp) from seven highly conserved housekeeping genes (*adk*, *atpG*, *frdB*, *fucK*, *mdh*, *pgi* and *recA*), the results are comparable to that of 16S rRNA analysis (Meats et al., 2003). The advantage of using multiple housekeeping genes to study *H. influenzae* strain relatedness is that they are highly conserved and in some cases absent from closely related *Haemophilus* species, which increases the strength of the analysis (Meats et al., 2003). As a result MLST is more routinely used in studies investigating the genetic relatedness of individual *H. influenzae* strains (Erwin et al., 2008), rather than in the differentiation of *H. influenzae* from *H. haemolyticus*.

However, it can be difficult to compare some MLST studies, as some authors do not use all seven housekeeping genes in their analysis. Furthermore some authors have recently characterised some *H. influenzae* isolates that completely lack the fucose operon (which contains the *fucK* gene), which hampers the discriminative power of including the *fucK* gene in the MLST scheme (Shuel et al., 2011). As such, it is important to recognise that the MLST scheme cannot be used to identify *H. haemolyticus* isolates instead it is solely used to confirm *H. influenzae* identity and infer phylogenetic relationships between these isolates.

2.6.2.3 PCR Targets

Numerous different PCR targets are currently used to differentiation *H. influenzae* from *H. haemolyticus* in the laboratory, and the majority of these targets are *H. influenzae* specific genes, including; *omp P6*, *fucK*, *hpd*, *hap*, *IgA* as well as the housekeeping genes of the MLST scheme. These genes appear to be good targets for *H. influenzae*, particularly NTHI identification, as they tend to be absent in isolates of *H. haemolyticus*. Conversely, there is only a single *H. haemolyticus* specific gene target; the *sodC* gene (Fung et al., 2006; Norskov-Lauritsen, 2009). Although, *sodC* is the primary positive genetic target for *H. haemolyticus* identification, it has also been more recently identified in a small number of NTHi isolates (Fung et al., 2006; Norskov-Lauritsen, 2009). To date, the effectiveness of these PCR targets at accurately discriminating between NTHi and non-heamolytic isolates of *H. haemolyticus* has been limited, such that many authors now prefer to use multiple genetic targets to increases the discriminative power of their PCR

algorithms (See Table 2.5) (Sandstedt et al., 2008; Wang et al., 2011; Theodore et al., 2012; Binks et al., 2012). Furthermore, as the clinical significance of isolating *H. haemolyticus* from a disease state is currently undereview, numerous authors are evaluating new PCR based approaches for NTHi and *H. haemolyticus* separation.

2.7 Chapter Summary

Over the past two decades altered PBP3 mediated resistance to β-lactam antibiotics, particularly ampicillin and amoxicillin, has emerged as an important mechanism of resistance in clinical isolates of *H. influenzae*. Based on the collective findings of previous studies BLNAR mediated resistance has been identified to account for between 15 to 50%, depending on the country, of all *H. influenzae* isolates encountered in the diagnostic laboratory. This alarmingly high prevalence prevents as a significant global concern, as therapeutic failures have been reported in cases of respiratory illness as well as meningitis when β-lactam antibiotics have been prescribed to treat *H. influenzae* infections.

The collective findings of previous phenotypic and genotypic characterisation studies have identified that the majority of these BLNAR isolates harbour low ampicillin MICs (0.5 - 2 μg/mL) that infer reduced susceptibility and not resistance, which makes them difficult to detect in the diagnostic laboratory using standard susceptibility testing techniques. Furthermore, variations in the definitions and methodologies (PCR vs. *ftsI* sequence) used to define BLNAR isolates by genotype have made the detection of BLNAR isolates in both clinical and surveillance studies difficult and unreliable.

With the recent rapid global dissemination of BLNAR isolates, and the recognition of the natural competency of the *Haemophilus* species for DNA uptake, some authors have postulated that resistance may be exchanged via HGT of the mutated *ftsI* genes.

Currently, there are many gaps in the literature regarding our basic understanding of the molecular evolution, epidemiology and clinical significance of this resistance mechanism in *H. influenzae*, such that the specific aims of research studies presented in this thesis were to:

- To molecularly characterise the *ftsI* gene and encoded PBP3 sequence profiles of both BLNAS and BLNAR isolates of *H. influenzae* (Chapters 3 and Chapter 4).
- To evaluate the molecular PCR methods currently used in BLNAR detection (Chapters 3 and 5).
- To determine the β-lactam susceptibility profiles, (both phenotypically and genotypically) of the close phylogenetic relative of *H. influenzae*; *Haemophilus haemolyticus* (Chapter 4).
- To look for a potential link between the *ftsI* genes of *H. influenzae* and *H. haemolyticus* and the dissemination of BLNAR mediated resistance, by specifically evaluating the ability of the two species to exchange and recombine *ftsI* genes both *in vivo* (Chapter 6) and *in vitro* (Chapter 7) settings.

These chapters have been removed for copyright or proprietary reasons.

Chapter 3 – SNP Based PCR Detection. Pages 75-90

Witherden EA, Kunde D, Tristram SG (2012). An evaluation of SNP-based PCR methods for the detection of β-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *J Infect Chemother*; 18 (4): 451-455.
http://dx.doi.org/10.1007/s10156-011-0356-5

Chapter 4 - β-lactam Resistance in *H. haemolyticus.* Pages 91-107

Witherden EA, and Tristram SG (2013). Prevalence and mechanisms of β-lactam resistance in *Haemophilus haemolyticus*. *J Antimicrob Chemother*; 68 (5): 1049-1053.
http://dx.doi.org/10.1093/jac/dks532

Chapter 5 - N526K SNP PCR Assay. Pages 108-124

Witherden EA, Kunde D, Tristram SG (2013). PCR screening for the N526K substitution in isolates of *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother*; 68 (10): 2255-2258.
http://dx.doi.org/10.1093/jac/dkt189

Chapter 6 - Mosaic *ftsI* genes. Pages 125-152

Witherden EA, Bajanca-Lavado MP, Tristram SG, Nunes A (2014). The role of inter-species recombination of the *ftsI* gene on the dissemination of altered penicillin-binding protein 3 mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother.* In Press
http://dx.doi.org/10.1093/jac/dku022

Chapter 7.0 – Manuscript 5

Recombination of *ftsI* genes among *Haemophilus influenza*e and *Haemophilus haemolyticus* isolates *in vitro*.

Authors: Elizabeth Witherden[1] and Stephen Tristram[1]

[1] *School of Human Life Sciences, University of Tasmania, Launceston, Tasmania, Australia.*

An edited version of this chapter will shortly be submitted for publication as an original research article.

7.1 Abstract

Objectives: To investigate the role of horizontal *ftsI* gene transfer among *Haemophilus influenzae* and *Haemophilus haemolyticus* isolates on the formation of mosaic *ftsI* genes, and the dissemination of the β-lactamase-negative ampicillin-resistant genotype within and between the *Haemophilus* species.

Methods: β-lactamase-negative ampicillin-susceptible (BLNAS) isolates *H. influenzae* Rd and *H. haemolyticus* ATCC 33390 were transformed by electroporation with PCR amplified *ftsI* genes from N526K-positive β-lactamase-negative ampicillin-resistant (BLNAR) isolates of non-typeable *H. influenzae* (NTHi) and *H. haemolyticus*. Successful transformants were selected at random from post-transformation growth on selective chocolate agar plates containing 0.5 μg/mL of ampicillin, and were tested for ampicillin susceptibility and full *ftsI* gene characterisation. Bioinformatic recombination analysis was performed on the *ftsI* gene sequences of all transformants in order to elucidate the degree of mosaic gene formation and the position of recombination events.

Results: We successfully transformed the ampicillin susceptible (N526) isolates *H. influenzae* Rd and *H. haemolyticus* ATCC 33390 to the ampicillin resistant N526K-positive BLNAR genotype.

Conclusion: Using *in vitro* transformation experiments we have shown that inter-species and intra-species recombination of the *ftsI* gene frequently occurs between *H. influenza*e and *H. haemolyticus* isolates, and that both mosaic *ftsI* gene formation and *de novo* mutational events play essential roles in the dissemination and

diversification of *ftsI* genes and PBP3 substitution profiles of BLNAR isolates of both species.

7.2 Introduction

Horizontal gene transfer (HGT) and homologous recombination contributes to the genetic evolution of bacterial populations, and are responsible for the dissemination of antibiotic resistance genes in many naturally competent bacterial species (Dowson et al., 1989; Smith-Vaughan et al., 1997; Kuhnert and Christensen, 2008). In the commensal bacterial flora of the human respiratory tract, resistance to penicillin and other β-lactam antibiotics mediated by altered penicillin-binding proteins (PBPs) has been shown to spread via the horizontal transfer and recombination of PBP encoding genes (Hakenbeck, 1998; Maiden, 1998; Hakenbeck et al., 2001). For example, the horizontal transfer and recombination of the *pbp2b* and *pbp2x* genes among commensal *Streptococcus* species, has lead to the dissemination of mosaic *pbp2b* and *pbp2x* genes that produce penicillin resistance in the respiratory pathogen *Streptococcus pneumoniae* (Hakenbeck et al., 2001). In a similar fashion, genetic recombination has been shown to play an important role in the development and dissemination of β-lactam resistance in other respiratory pathogens including both pathogenic and commensal *Neisseria* species, and more recently *Haemophilus influenzae* (Spratt et al., 1992; Takahata et al., 2007; Witherden et al., 2014).

β-lactamase-negative ampicillin-resistance (BLNAR) in *H. influenzae* has been attributed to a reduction in the binding affinity of penicillin-binding protein 3 (PBP3)

for β-lactam antibiotics (Ubukata et al., 2001). Originally, these altered PBP3's were thought to arise through the acquisition of *de novo* point mutations in the chromosomally located *ftsI* gene (Ubukata et al., 2001; Kubota et al., 2006; Hotomi et al., 2007). These mutations infer key amino acid changes in the transpeptidase domain of PBP3 such as the D350N, R517H, N526K, S385T, L389F and A502T/V substitutions that have been linked to ampicillin resistance (Ubukata et al., 2001; Osaki et al., 2005; Sanbongi et al., 2006; Skaare et al., 2010). Of particular importance is the N526K substitution, as it lines the active site pocket of the mature PBP3 protein inferring low-level resistance to ampicillin and currently defines the resistance genotype of BLNAR isolates of *H. influenzae* (Ubukata et al., 2001).

Over the past two decades the prevalence of BLNAR mediated resistance has risen rapidly worldwide, and now accounts for up to 25-30% of β-lactam resistant isolates of *H. influenzae* in many countries (Tristram et al., 2007). With this rapid dissemination of the BLNAR genotype, many authors have documented the presence of identical *ftsI* gene sequences among clinically and geographically distinct BLNAR isolates, suggesting that horizontal transfer of the *ftsI* gene may be contributing to the rapid spread of the BLNAR genotype (Takahata et al., 2007; Skaare et al., 2010; Barbosa et al., 2011).

In 2007, Takahata and colleagues provided evidence for the horizontal transfer and recombination of the *ftsI* gene among BLNAR isolates of *H. influenzae*, and identified the presence of mosaic *ftsI* gene structures in several clinical BLNAR isolates (Takahata et al., 2007). Characterisation of these mosaic *ftsI* structures identified that distinct *ftsI* gene fragments had been inherited from the respiratory

tract commensal *H. haemolyticus* (Takahata et al., 2007). Witherden and colleagues (2014) built on the work of Takahata et al. (2007) by characterising the *ftsI* gene sequences of 100 clinical non-typeable *H. influenzae* (NTHi) and *H. haemolyticus* isolates. In that study, the authors described that 36% (18/50) of NTHi and 30% (15/50) of *H. haemolyticus* isolates harboured mosaic *ftsI* genes, that resulted from the inter-species recombination of the *ftsI* gene between the two species (Witherden et al., 2014). Interestingly, these mosaic *ftsI* gene structures were exclusively restricted to isolates harbouring the N526K-positive BLNAR genotype. Thus highlighting the important role recombination of the *ftsI* gene has on the dissemination of BLNAR mediated resistance (Witherden et al., 2014).

In order to gain a better understanding of *ftsI* gene recombination events, we set out to characterise those homologous recombination events that result in the transmission of resistant *ftsI* genes between NTHi and *H. haemolyticus* species, *in vitro*.

7.3 Materials and Methods

7.3.1 Bacterial Isolates

Both recipient and donor isolates were selected from the UTAS culture collection. β-lactamase-negative ampicillin-susceptible (BLNAS) N526 control isolates *H. influenzae* Rd and *H. haemolyticus* ATCC 33390 were used as the transformation recipients, whilst four isolates (two *H. influenzae* and two *H. haemolyticus* isolates) were selected as transformation donors. All transformation donors were N526K-

positive BLNAR isolates with relatively high MICs to ampicillin and were previously isolated from clinical samples (Witherden and Tristram, 2013; Witherden et al., 2014). The specific characteristics of the donor and recipient isolates used in the transformation experiments are given in Table 7.1.

7.3.2 Transformation Experiments

Both intra-species and inter-species transformation experiments were performed, such that each recipient was transformed by electroporation with PCR amplified *ftsI* genes from each donor. This resulted in two intra-species and two inter-species recombination experiments per recipient.

7.3.2.1 Preparation of Donor DNA

The entire 1833 bp open reading frame (ORF) of the *ftsI* gene as well as ~317 bp up-stream and ~569 bp down-stream flanking regions were amplified from each donor isolate as previously described (Takahata et al., 2007). The resultant ~ 2719 bp PCR product was purified using the Qiagen PCR clean-up kit (Qiagen, Victoria, Australia) in accordance with the manufactures instructions and had the concentration of DNA quantified spectrophotometrically prior to being stored at -20^{o}C until use.

Table 7.1 Characteristics of transformation donor and recipients.

Isolates		AMP MIC (µg/mL)	Position of important amino acid substitutions in PBP3[c]												
			239	**274**	**350**	**352**	**357**	**377**	**385**	**389**	**449**	**502**	**526**	**547**	**569**
H. influenzae Rd	Recipient	0.125	**A**	**E**	**D**	**T**	**S**	**M**	**S**	**L**	**I**	**A**	**N**	**V**	**N**
H. influenzae Isolate UTAS 215[a]	Donor	3	E										K	I	S
H. influenzae Isolate UTAS 252[b]	Donor	0.5	E		N		N	I	T	F			K	I	S
H. haemolyticus ATCC 33390	Recipient	0.19	**E**	**D**	**N**	**G**	**S**	**I**	**S**	**L**	**I**	**A**	**N**	**I**	**S**
H. haemolyticus Isolate L23	Donor	2		E		T						V	K		
H. haemolyticus Isolate L48	Donor	1.5									V		K		

[a] UTAS 215 is CLSI *H. influenzae* susceptibility control strain ATCC 49247 (BLNAR).

[b] UTAS 252 is from Ubukata et al. (2001) and is termed high-BLNAR isolate ME870 in that study.

[c] The deduced PBP3 sequences of the *H. influenzae* donor isolates were compared to that of *H. influenzae* Rd, whilst the deduced PBP3 amino acid sequence of *H. haemolyticus* donors was compared to the *H. haemolyticus* baseline control strain *H. haemolyticus* ATCC 33390.

7.3.2.2 Preparation of competent recipient cells

Recipient isolates *H. influenzae* Rd and *H. haemolyticus* ATCC 33390 were grown in supplemented brain heart infusion broth (sBHI) for 4-5 hours at 37°C until an optical density (OD_{650}) of 0.5 was reached (Ubukata et al., 2001).

Cells were chilled on ice for 30 minutes, and harvested by centrifugation (4200 x g) for 20 minutes at 4^{o}C. Harvested cells were thoroughly washed four times in phosphate-buffered saline glucose (PSG) buffer, before the final cell pellet was suspended in 1.0 ml of cold PSG, and stored at -80°C until use (Ubukata et al., 2001).

7.3.2.3 Electroporation

A total of 5 µL (approximately 100 ng) of purified *ftsI* PCR product was added to 40 µL of ice-chilled PSG washed competent cells. The transformation mixture was allowed to stand on ice for 1 min prior to electroporation using the GENE PULSER II electroporation apparatus (Bio-Rad, Hercules, CA, U.S). Electroporation was performed using chilled 2 mm gap cuvettes and the following conditions: 2.5 kV, and 25 mF with time constants of 4.7 to 4.8 ms.

Post-electroporation, cells were mixed with 1.0 mL of pre-warmed sBHI broth and incubated for 4 hours at 37^{o}C before plating out 10 µL of cells (diluted to 100 µL with sBHI) on to selective chocolate agar plates containing 0.5 µg of ampicillin. After overnight incubation in 5-10% CO_2 at 37^{o}C the number of colony forming units (CFUs) per plate were calculated.

For each experiment, 10 individual colonies were selected at random and screened for the presence of the BLNAR-defining N526K substitution using the real-time PCR protocol previously described (Witherden et al., 2013).

7.3.3 Analysis of Transformants

A total of five N526K-positive PCR confirmed transformants were selected per transformation experiment, and were subjected to sequencing of the entire *ftsI* gene for confirmation as previously described (Witherden and Tristram, 2013). Transformants also underwent E-tests for susceptibility to ampicillin according to the manufactures instructions (BioMerieux, NSW, Australia). Bioinformatics analysis was performed on the *ftsI* gene and adjacent flanking regions of all transformants and the location and position of all recombination events were characterised using the methodologies previously described (Witherden et al., 2014).

7.4 Results and Discussion

7.4.1 Transformation Experiments

BLNAS isolates *H. influenzae* Rd and *H. haemolyticus* ATCC 33390 were successfully transformed with PCR amplified *ftsI* gene products (~2719 bp) from N526K-positive *H. influenzae* BLNAR isolates U252 and U215 as well as N526K-positive *H. haemolyticus* BLNAR isolates L23 and L48. The transformation frequency ranged from $1x10^{-6}$ to $4x10^{-6}$ for all experiments and there was no apparent difference in the transformation frequencies between the intra-species and inter-species

transformation experiments of either recipient. Characteristics of the resultant transformants are given in Table 7.2 and Table 7.3, respectively.

All transformants had elevated MICs to ampicillin compared to their respective parental recipients, and this correlated with the successful transmission of the N526K BLNAR-defining substitution (Table 7.2; Table 7.3). Differences in the ampicillin MICs of transformants from a single experiment was frequently noted and this appeared to be due to the size of the recombinant fragment inherited from the donor and the number and position of deduced PBP3 substitutions inherited with the gene fragment (Table 7.2; Table 7.3). Furthermore, differences in ampicillin MICs were identified in some donor/recipient combinations where identical *ftsI* gene fragments were transferred, and this variation could be attributed to the inherent imprecision of the E-test methodology for MIC determination.

Variations in the size and position of the gene fragment transferred between the donors and recipients were frequently observed in both the intra-species and inter-species transformation experiments. For example, transformants from a single experiment inherited either identical or diverse *ftsI* gene fragments and this influenced the resultant amino acid substitutions in PBP3. Specific analysis of the five transformants obtained from the *H. influenzae* Rd /*H. haemolyticus* L23 transformation experiment, identified that 3 of the transformants (7A, 7H and 7G) had *ftsI* gene sequences and PBP3 profiles identical to that of the donor, whilst two isolates (7B and 7C) inherited partial *ftsI* gene fragments and heterogeneous PBP3 substitutions profiles, that varied from that of the original donor (Table 7.2).

Table 7.2 Characteristic of *H. influenzae* transformants.

			Substitutions in PBP3													
Experiment	**Transformant**	**AMP MIC**	**239**	**274**	**350**	**352**	**357**	**377**	**385**	**389**	**449**	**502**	**526**	**547**	**569**	**Other**
Intra-species Transformations																
1.	**Rd** (Recipient)	**0.125**	**A**	**E**	**D**	**T**	**S**	**M**	**S**	**L**	**I**	**A**	**N**	**V**	**N**	
	U252 (Donor)	**0.5**	**E**		**N**		**N**	**I**	**T**	**F**			**K**	**I**	**S**	
	3B	0.75	E		N		N	I	T	F			K	I	S	
	3C	0.5	E		N		N	I	T	F			K	I	S	M503V
	3E	0.75	E		N		N	I	T	F			K	I	S	
	3F	0.75	E		N		N	I	T	F			K	I	S	
	3G	0.5	E		N		N	I	T	F			K	I	S	
2.	**Rd (**Recipient)	**0.125**	**A**	**E**	**D**	**T**	**S**	**M**	**S**	**L**	**I**	**A**	**N**	**V**	**N**	
	U215 (Donor)	**3**	**E**										**K**	**I**	**S**	**S273A, A554T**
	5A	0.38	E										K	I	S	S273A, A554T, D583G
	5B	0.38	E										K	I	S	S273A, A554T,
	5J	0.25	E										K	I	S	S273A, A554T,
	5H		E										K	I	S	S273A, A554T,
	5D	0.38	E										K	I	S	S273A, I375T, A554T
Inter-species Transformations																
3.	**Rd (**Recipient)	**0.125**	**A**	**E**	**D**	**T**	**S**	**M**	**S**	**L**	**I**	**A**	**N**	**V**	**N**	
	L23 (Donor)	**2**	**E**		**N**			**I**				**V**	**K**	**I**	**S**	**S242A,**
	7A	0.38	E		N			I				V	K	I	S	S242A, N305D, V479A
	7B	0.38			N			I				V	K	I	S	
	7C	0.25						I				V	K	I	S	
	7H	0.5	E		N			I				V	K	I	S	S242A,
	7G	0.5	E		N			I				V	K	I	S	S242A,
4.	**Rd** (Recipient)	**0.125**	**A**	**E**	**D**	**T**	**S**	**M**	**S**	**L**	**I**	**A**	**N**	**V**	**N**	
	L48 (Donor)	**1.5**	**E**	**D**	**N**	**G**		**I**			**V**		**K**	**I**	**S**	**S242A, K344R, K355T, L356V**
	9B	0.38	E	D	N	G		I			V		K	I	S	S242A, K344R, K355T, L356V
	9D	0.5									V		K	I	S	
	9E	0.38									V		K	I	S	
	9G	0.38		D	N	G		I			V		K	I	S	K344R, K355T, L356V, K553I
	9H	0.25			N			I			V		K	I	S	K344R, K355T, L356V, A557V

Table 7.3 Characteristic of *H. haemolyticus* transformants.

			Substitutions in PBP3													
Experiment	**Transformant**	**AMP MIC[a]**	**239**	**274**	**350**	**352**	**357**	**377**	**385**	**389**	**449**	**502**	**526**	**547**	**569**	**Other**
Intra-species Transformations																
1.	**Hh** (Recipient)	**0.19**	**E**	**D**	**N**	**G**	**S**	**I**	**S**	**L**	**I**	**A**	**N**	**I**	**S**	
	L23 (Donor)	**2**		**E**		**T**						**V**	**K**			**R344K, T355K, V356L**
	Ca	1		E		T						V	K			T355K, V356L
	Cc	0.75		E		T						V	K			R344K, T355K, V356L
	Cd	0.5		E		T						V	K			K245E, A249V, R344K, T355K, V356L, V364I
	Ce	1				T						V	K			R344K, T355K, V356L
	Cf	0.75										V	K			T355K, V356L
2.	**Hh** (Recipient)	**0.19**	**E**	**D**	**N**	**G**	**S**	**I**	**S**	**L**	**I**	**A**	**N**	**I**	**S**	
	L48 (Donor)	**1.5**									**V**		**K**			
	Da	0.5									V		K			
	Db	1									V		K			
	Dc	0.75									V		K			
	De	0.75									V		K			
	Dj	0.38									V		K			A544V
Inter-species Transformations																
3.	**Hh** (Recipient)	**0.19**	**E**	**D**	**N**	**G**	**S**	**I**	**S**	**L**	**I**	**A**	**N**	**I**	**S**	
	U252 (Donor)	**0.5**		**E**		**T**	**N**		**T**	**F**			**K**			**A242S, R344K, T355K, V356L**
	Aa	2		E		T	N		T	F			K			R344K, T355K, V356L
	Ab	1.5				T	N		T	F			K			R344K, T355K, V356L
	Af	2		E		T	N		T	F			K			A242S, R344K, T355K, V356L
	Ag	2				T	N		T	F			K			R344K, T355K, V356L
	Ah	4		E		T	N		T	F			K			R344K, T355K, V356L, E505K
4.	**Hh** (Recipient)	**0.19**	**E**	**D**	**N**	**G**	**S**	**I**	**S**	**L**	**I**	**A**	**N**	**I**	**S**	
	U215 (Donor)	**3**		**E**	**D**	**T**		**M**					**K**			A242S, S273A, R344K, T355K, V356L, A554T
	Ba	1		E	D	T		M					K			A242S, S273A, R344K, T355K, V356L, A554T
	Bb	1											K			A554T
	Bc	1											K			A554T
	Bd	1.5			D	T		M					K			R344K, T355K, V356L, A554T
	Bg	1.5											K			A554T

[a] Ampicillin MIC in µg/mL

This highlights the fact that multiple amino acid substitutions can be transferred between isolates during a single recombination event. This finding may be why identical PBP3 substitution profiles (and *ftsI* gene sequences) are frequently reported in genetically and geographically diverse BLNAR isolates (Osaki et al., 2005; Skaare et al., 2010).

Moreover detailed analysis of the *ftsI* gene sequences of transformants identified the presence of new *de novo* mutations in the *ftsI* gene of some transformants (not present in either donor or recipient) that resulted in previously unreported substitutions in PBP3. This data supports the notion that point mutations occur within the *ftsI* gene and this mechanism plays an important role in the diversification of *ftsI* and PBP3 sequences.

In summary, by using *in vitro* transformation experiments we have shown that inter-species and intra-species recombination of the *ftsI* gene frequently occurs between *H. influenza*e and *H. haemolyticus* isolates, and that both mosaic *ftsI* gene formation and *de novo* mutational events play essential roles in the dissemination and diversification of *ftsI* genes and PBP3 substitution profiles of BLNAR isolates of both species.

7.4.2 Recombination Analysis of Transformants

Recombination analysis was performed on all transformants to assess the position and size of the respective recombinant fragments. In general successful transformation of an isolate resulted from either the inheritance of the entire *ftsI*

gene from the respective donor, or more commonly a partial *ftsI* gene fragment. The exact position of the crossover event where the origin of the *ftsI* gene changed from recipient to donor was calculated from the informative sites generated by SimPlot analysis. Essentially the position of crossover locations was calculated as the DNA fragment that sits between the last informative site (last polymorphism) supporting the recipient as the origin of the *ftsI* gene, and the first informative site (first polymorphism) supporting the donor as the origin, respectively.

This study uses known parental isolates to evaluate recombination events, and this is advantageous for the detection of potential intra-species recombination events. This is important because our current understanding about the ability of *H. influenzae* isolates to undergo intra-species *ftsI* gene recombination is inferred from the natural competence of NTHi isolates for recombination, and the reports of inter-species recombination of the *ftsI* gene among *Haemophilus* species. As a result previous studies using clinical isolates have had difficulty characterising intra-species recombination events due to the high degree of homology between the *ftsI* gene sequences of isolates from the same species.

The exact size of the crossover location differed between the intra-species and inter-species transformation experiments of both recipients, and tended to be smaller in the inter-species transformants. This can be attributed to the higher fixation of polymorphisms when *ftsI* genes from *H. influenzae* and *H. haemolyticus* are compared. This makes it easier to find a point of difference between the two sequences that infers a phylogenetic origin. Finally, as the position and size of the recombinant fragment frequently varied between the donor/recipient

transformation combinations, common recombination hotspots were identified within the *ftsI* gene of some transformants, suggesting that recombination of the *ftsI* gene is not always a random event.

7.4.2.1 Recombination in *H. influenzae* Transformants

The position and size of the recombinant fragment inherited from the *H. influenzae* BLNAR donors in the *H. influenzae* inter-species transformants are shown in Table 7.4 and Figure 7.1 whilst results from the intra-species transformations are given in Table 7.5 and Figure 7.2. In the intra-species *H. influenzae* transformation experiments, most of the transformants inherited partial *ftsI* genes from the *H. influenzae* BLNAR donors, whilst only three transformants (5H, 5J and 5D) inherited the entire *ftsI* gene (Table 7.3, Figure 7.2). All the *H. influenzae* inter-species transformants inherited partial *ftsI* gene fragments from the *H. haemolyticus* BLNAR donors (Table 7.5, Figure 7.3).

7.4.2 Transformation in *H. haemolyticus*

The position and size of the recombinant fragment inherited from the *H. haemolyticus* BLNAR donors in the *H. haemolyticus* inter-species transformants are shown in Table 7.4 and Figure 7.4 whilst results from the intra-species transformants are given in Table 7.5 and Figure 7.2. Genetic recombination of the *ftsI* gene in the intra-species *H. haemolyticus* transformation experiments resulted in mosaic *ftsI* genes where all transformants inherited partial *ftsI* gene fragments

from the BLNAR donors. Although the exact position of the recombination fragment differed in size and location between the transformants characterised, a number of transformants shared at least one crossover region. This suggests that some segments of the *ftsI* gene are more susceptible to recombination events.

Common recombination hotspots were identified within the transformants from the intra-species transformation experiments. Both transformants Cc and Db inherited a recombinant fragment position between 133 to 1716 bp of the *ftsI* gene from their respective donors. Furthermore, transformants Da and Dc shared a single crossover event, resulting in the generation of different sized recombination fragments starting from the same position in the *ftsI* gene, 396to 1833 bp (Da) and 396 to 1485 bp (Dc).

Table 7.4 Recombination analysis performed on *H. influenzae* Rd intra-species transformants.

	MIC (µg/mL)	Crossover Regions[b]				Recombinant Fragment within the *ftsI* gene[d]		
Transformants[a]	AMP	(n)	Position (bp)[c]	Location	*P*-value	Size (bp)	Position in *ftsI* (bp)	Position in PBP3 (aa)
Rd/U252								
3B	0.75	1	421 to 863	Within *ftsI*	<0.0001	969	864-1833	288-611
3C	0.5	2	-99 to 166 (L)	Upstream flanking + within *ftsI*	0.0004	1548	167-1715	56-572
			1716 to +92 (R)	Within *ftsI* + Downstream flanking	0.004			
3E	0.75	2	421 to 863 (L)	Within *ftsI*	<0.0001	969	864-1833	288-611
			+137 to +283 (R)	Downstream flanking region	0.0556			
3F	0.75	2	421 to 863 (L)	Within *ftsI*	<0.0001	969	864-1833	288-611
			+137 to +283 (R)	Downstream flanking region	0.0556			
3G	0.5	2	-99 to 166 (L)	Upstream flanking + within *ftsI*	0.0003	1666	167-1833	56-611
			+137 to +229 (R)	Downstream flanking region	0.0031			
Rd/U215								
5A	0.38	2	163 to 453 (L)	Within *ftsI*	0.0476	1379	454-1833	152-611
			+177 to +283 (R)	Downstream flanking region	0.0043			
5B	0.38	2	163 to 453 (L)	Within *ftsI*	0.0476	1379	454-1833	152-611
			+177 to +283 (R)	Downstream flanking region	0.0043			
5J	0.25	1	+58 to +64	Downstream flanking region	<0.001	1833	1-1833	1-611
5H		1	+177 to +283	Downstream flanking region	0.0109	1833	1-1833	1-611
5D	0.38	1	+58 to +64	Downstream flanking region	<0.0001	1833	1-1833	1-611

[a] Transformants, Recipient/donor; Rd, *H. influenzae* Rd; U252, *H. influenzae* UTAS 252; U215, *H. influenzae* UTAS 215.

[b] Data for the crossover regions is calculated from the SimPlot and BootScan analysis using the *ftsI*+flanking regions contigs using *H. parahaemolyticus* as an out-group; (n), number of crossover regions identified; position, position of the crossover region is relative to the bp numbering of the *ftsI* ORF.

[c] (L), denotes the first crossover site to the left; (R), denotes the second crossover site on the right.

[d] Size, size of recombinant fragment (bp); Position in *ftsI*, position of the recombinant fragment relative to the ORF of *ftsI* (bp); Position in PBP3, deduced position of the recombinant fragment in the encoded PBP3 protein.

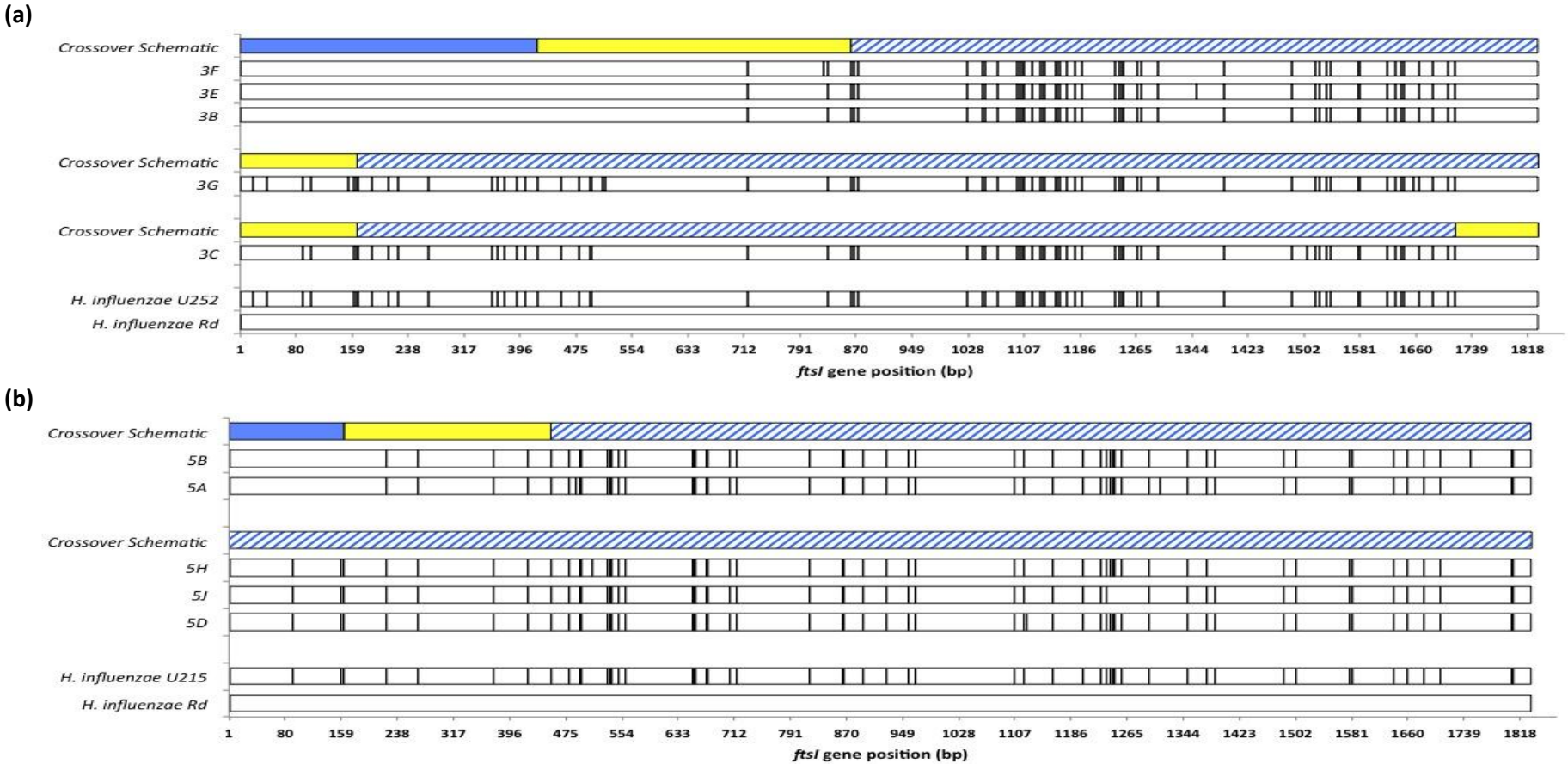


Figure 7.1 Mosaic *ftsI* gene structures identified in the *H. influenzae* intra-species transformants.

Schematic representations of the divergence in the *ftsI* gene on transformants compared to the *ftsI* sequence of recipient isolate *H. influenzae* Rd. Solid black lines, denote the position of the nucleotides that differ from the corresponding nucleotide in the reference sequence. Crossover schematics show the proportion of the *ftsI* gene most similar to the donor or recipient; Solid blue shading, most similar to recipient DNA from *H. influenzae* Rd; Solid yellow shading, crossover location; Hashed blue shading, most similar to donor DNA from *H. influenzae* U215 or U252. **(a).** Depicts the Rd/U252 transformants. **(b).** Depicts the Rd/U215 transformants.

Table 7.5 Recombination analysis performed on *H. influenzae* Rd inter-species transformants.

	MIC (µg/mL)	Crossover Regions[b]				Recombinant Fragment within the *ftsI* gene		
Transformants[a]	**AMP**	**(n)**	**Position (bp)[c]**	**Location**	***P*-value**	**Size (bp)**	**Position in *ftsI* (bp)**	**Position in PBP3 (aa)**
Rd/L23								
7A	0.38	2	622 to 662 (L)	Within *ftsI*	<0.0001	1095	663-1758	221-586
			1759 to +91 (R)	Within *ftsI*+Downstream flanking	<0.0001			
7B	0.38	2	724 to 950 (L)	Within *ftsI*	<0.0001	807	951-1758	317-586
			1759 to +269 (R)	Within *ftsI*+Downstream flanking	0.0058			
7C	0.25	2	1054 to 1103 (L)	Within *ftsI*	<0.0001	654	1104-1758	368-586
			1759 to +269 (R)	Within *ftsI*+Downstream flanking	0.0065			
7H	0.5	2	-35 to 13 (L)	Upstream flanking + within *ftsI*	<0.0001	1744	14-1758	5-586
			1759 to + 228 (R)	Within *ftsI*+Downstream flanking	<0.0001			
7G	0.5	2	-35 to 13 (L)	Upstream flanking + within *ftsI*	<0.0001	1744	14-1758	5-586
			1759 to +91 (R)	Within *ftsI*+Downstream flanking	<0.0001			
Rd/L48								
9B	0.38	2	519 to 557 (L)	Within *ftsI*	<0.0001	1200	558-1758	186-586
			1717 to +91 (R)	Within *ftsI* + Downstream flanking	<0.0001			
9D	0.5	1	1189 to 1265	Within *ftsI*	<0.0001	567	1266-1833	422-611
9E	0.38	1	1390 to 1484	Within *ftsI*	<0.0001	348	1485-1833	495-611
9G	0.38	1	763 to 821	Within *ftsI*	<0.0001	1011	822-1833	274-611
9H	0.25	2	895 to 956 (L)	Within *ftsI*	<0.0001	876	957-1833	319-611
			+71 to +164 (R)	Downstream flanking region	0.0026			

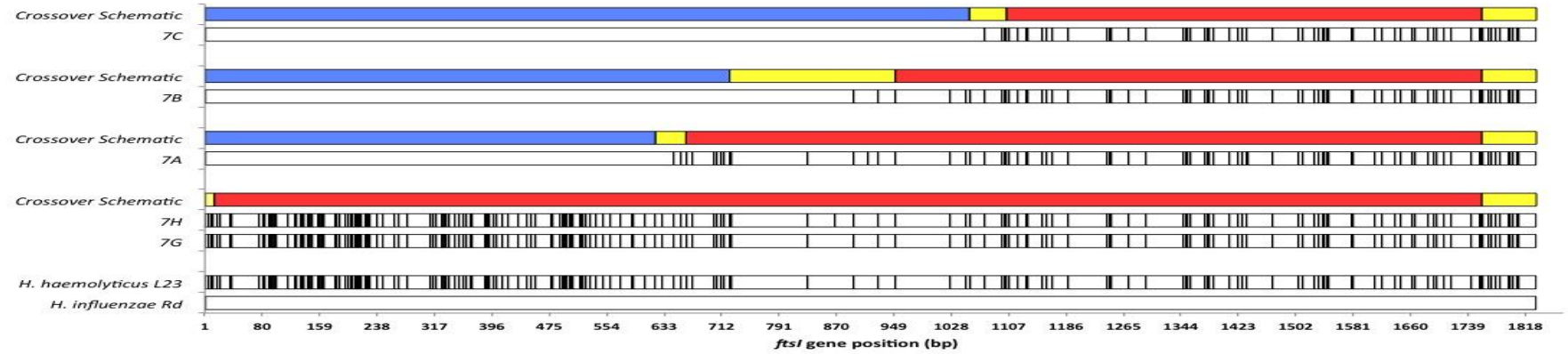
[a] Transformants, Recipient/donor; Rd, *H. influenzae* Rd; U252, *H. influenzae* UTAS 252; U215, *H. influenzae* UTAS 215.

[b] Data for the crossover regions is calculated from the SimPlot and BootScan analysis using the *ftsI*+flanking regions contigs using *H. parahaemolyticus* as an out-group; (n), number of crossover regions identified; position, position of the crossover region is relative to the bp numbering of the *ftsI* ORF.

[c] (L), denotes the first crossover site to the left; (R), denotes the second crossover site on the right.

[d] Size, size of recombinant fragment (bp); Position in *ftsI*, position of the recombinant fragment relative to the ORF of *ftsI* (bp); Position in PBP3, deduced position of the recombinant fragment in the encoded PBP3 protein.

(a)



(b)

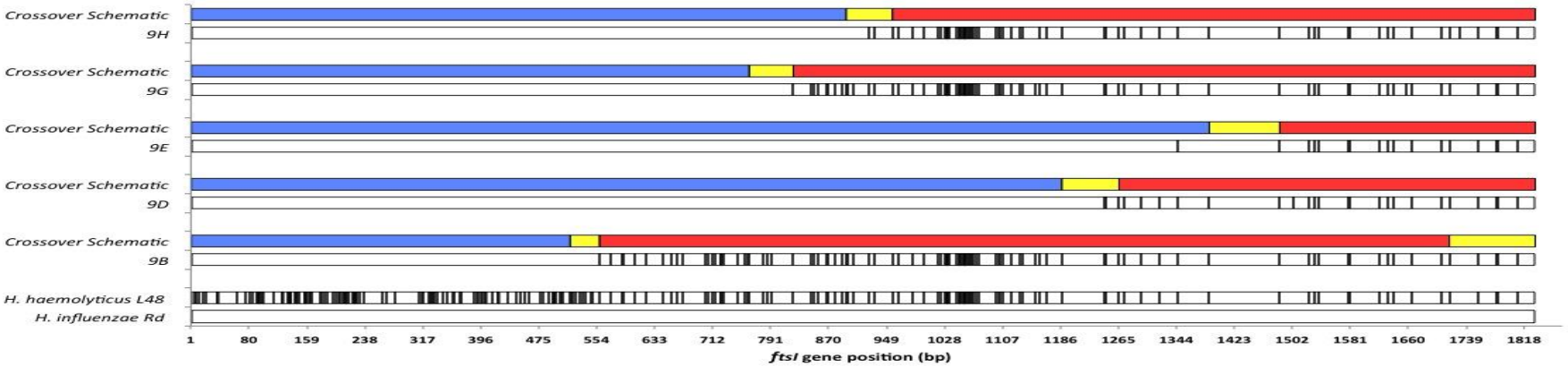


Figure 7.2 Mosaic *ftsI* gene structures identified in the *H. influenzae* recipient inter-species transformants.

Schematic representations of the divergence in the *ftsI* gene on transformants compared to the *ftsI* sequence of recipient isolate *H. influenzae* Rd. Solid black lines, denote the position of the nucleotides that differ from the corresponding nucleotide in the reference sequence. Crossover schematics show the proportion of the *ftsI* gene most similar to the donor or recipient; Solid blue shading, most similar to recipient DNA from *H. influenzae* Rd; Solid yellow shading, crossover location; Solid red shading, most similar to donor DNA from *H. haemolyticus* L23 or L48. **(a).** Depicts the Rd/L48 transformants. **(b).** Depicts the Rd/L48 transformants.

Table 7.6 Recombination analysis performed on *H. haemolyticus* ATCC 33390 intra-species transformants.

	MIC (µg/mL)	Crossover Regions[b]			Recombinant Fragment within the *ftsI* gene		
Transformants[a]	**AMP**	**(n)**	**Position (bp)**	**Location**	***P*-value**	**Size (bp)**	**Position in *ftsI* (bp)**	**Position in PBP3 (aa)**
Hh ATCC/L23								
Ca	1	1	463 to 559	Within *ftsI*	<0.0001	1273	560-1833	187-611
Cc	0.75	2	93 to 132 (L)	Within *ftsI*	<0.0001	1583	133-1716	45-572
			1717 to +58 (R)	Within *ftsI* + downstream flanking	<0.0001			
Cd	0.5							
Ce	1	2	958 to 998 (L)	Within *ftsI*	<0.0001	549	999-1548	333-516
			1549 to 1685 (R)	Within *ftsI*	<0.0001			
Cf	0.75	2	1069 to 1145 (L)	Within *ftsI*	<0.0001	687	1146-1833	382-611
			+164 to +229 (R)	Downstream flanking region	0.0014			
Hh ATCC/L48								
Da	0.5	1	214 to 395	Within *ftsI*	<0.0001	1437	396-1833	132-611
Db	1	1	-159 to 132	Upstream flanking + within *ftsI*	0.0026	1700	133-1833	45-611
Dc	0.75	2	214 to 395 (L)	Within *ftsI*	<0.0001	1089	396-1485	132-495
			1486 to 1661 (R)	Within *ftsI*	<0.0001			
De	0.75	2	631 to 830 (L)	Within *ftsI*	<0.0001	912	831-1743	277-581
			1744 to +191 (R)	Within *ftsI* + downstream flanking	0.0065			
Dj	0.38	2	847 to 1019 (L)	Within *ftsI*	<0.0001	723	1020-1743	340-581
			1744 to 1757 (R)	Within *ftsI*	0.0018			

[a] Transformants, Recipient/donor; HH ATCC, *H. haemolyticus* ATCC 33390; L23, *H. haemolyticus* L23; L48, *H. haemolyticus* L48.

[b] Data for the crossover regions is calculated from the SimPlot and BootScan analysis using the *ftsI*+flanking regions contigs using *H. parahaemolyticus* as an out-group; (n), number of crossover regions identified; position, position of the crossover region is relative to the bp numbering of the *ftsI* ORF.

[c] (L), denotes the first crossover site to the left; (R), denotes the second crossover site on the right.

[d] Size, size of recombinant fragment (bp); Position in *ftsI*, position of the recombinant fragment relative to the ORF of *ftsI* (bp); Position in PBP3, deduced position of the recombinant fragment in the encoded PBP3 protein.

Figure 7.3 Mosaic *ftsI* gene structures identified in the *H. haemolyticus* intra-species transformants.

Schematic representations of the divergence in the *ftsI* gene on transformants compared to the *ftsI* sequence of recipient isolate *H. influenzae* Rd. Solid black lines, denote the position of the nucleotides that differ from the corresponding nucleotide in the reference sequence. Crossover schematics show the proportion of the *ftsI* gene most similar to the donor or recipient; Solid red shading, most similar to recipient DNA from *H. haemolyticus* ATCC 33390; Solid yellow shading, crossover location; Hashed red shading, most similar to donor DNA from *H. haemolyticus* L23 or L48. **(a).** Depicts the HH ATCC 33390/L23 transformants. **(b).** Depicts the HH ATCC 33390/L48 transformants

Table 7.7 Recombination analysis performed on *H. haemolyticus* ATCC 33390 inter-species transformants.

	MIC (µg/mL)	Crossover Regions[b]				Recombinant Fragment within the *ftsI* gene		
Transformants[a]	**AMP**	**(n)**	**Position (bp)**	**Location**	***P*-value**	**Size (bp)**	**Position in *ftsI* (bp)**	**Position in PBP3 (aa)**
HH ATCC/U252								
Aa	2	1	781-830	Within *ftsI*	<0.0001	1002	831-1833	277-611
Ab	1.5	2	985 to 998 (L)	Within *ftsI*	<0.0001	834	999-1833	333-611
			+164 to +229 (R)	Downstream flanking region	0.0025			
Af	2	1	559 to 587	Within *ftsI*	<0.0001	1245	588-1833	196-611
Ag	2	2	880 to 893 (L)	Within *ftsI*	<0.0001	849	894-1743	298-581
			1744 to +58 (R)	Within *ftsI* +Downstream flanking	0.0003			
Ah	4	1	712 to 746	Within *ftsI*	<0.0001	1086	747-1833	249-611
Hh ATCC/U215								
Ba	1	2	229 to 260 (L)	Within *ftsI*	<0.0001	1482	261-1743	87-581
			1744 to +137 (R)	Within *ftsI* +Downstream flanking	<0.0001			
Bb	1	2	1384 to 1433 (L)	Within *ftsI*	<0.0001	309	1434-1743	478-581
			1744 to +191 (R)	Within *ftsI* +Downstream flanking	0.0002			
Bc	1	2	1468 to 1502 (L)	Within *ftsI*	<0.0001	330	1503-1833	501-611
			+177 to +226 (R)	Downstream flanking region	0.0008			
Bd	1.5	2	985 to 998 (L)	Within *ftsI*	<0.0001	834	999-1833	333-611
			+177 to +191 (R)	Downstream flanking region	<0.0001			
Bg	1.5	2	1267 to 1346 (L)	Within *ftsI*	<0.0001	486	1347-1833	449-1833
			+177 to +226 (R)	Downstream flanking region	<0.0001			

[a] Transformants, Recipient/donor; HH ATCC, *H. haemolyticus* ATCC 33390; U252, *H. influenzae* UTAS 252; U215, *H. influenzae* UTAS 215.

[b] Data for the crossover regions is calculated from the SimPlot and BootScan analysis using the *ftsI*+flanking regions contigs using *H. parahaemolyticus* as an out-group; (n), number of crossover regions identified; position, position of the crossover region is relative to the bp numbering of the *ftsI* ORF.

[c] (L), denotes the first crossover site to the left; (R), denotes the second crossover site on the right.

[d] Size, size of recombinant fragment (bp); Position in *ftsI*, position of the recombinant fragment relative to the ORF of *ftsI* (bp); Position in PBP3, deduced position of the recombinant fragment in the encoded PBP3 protein.

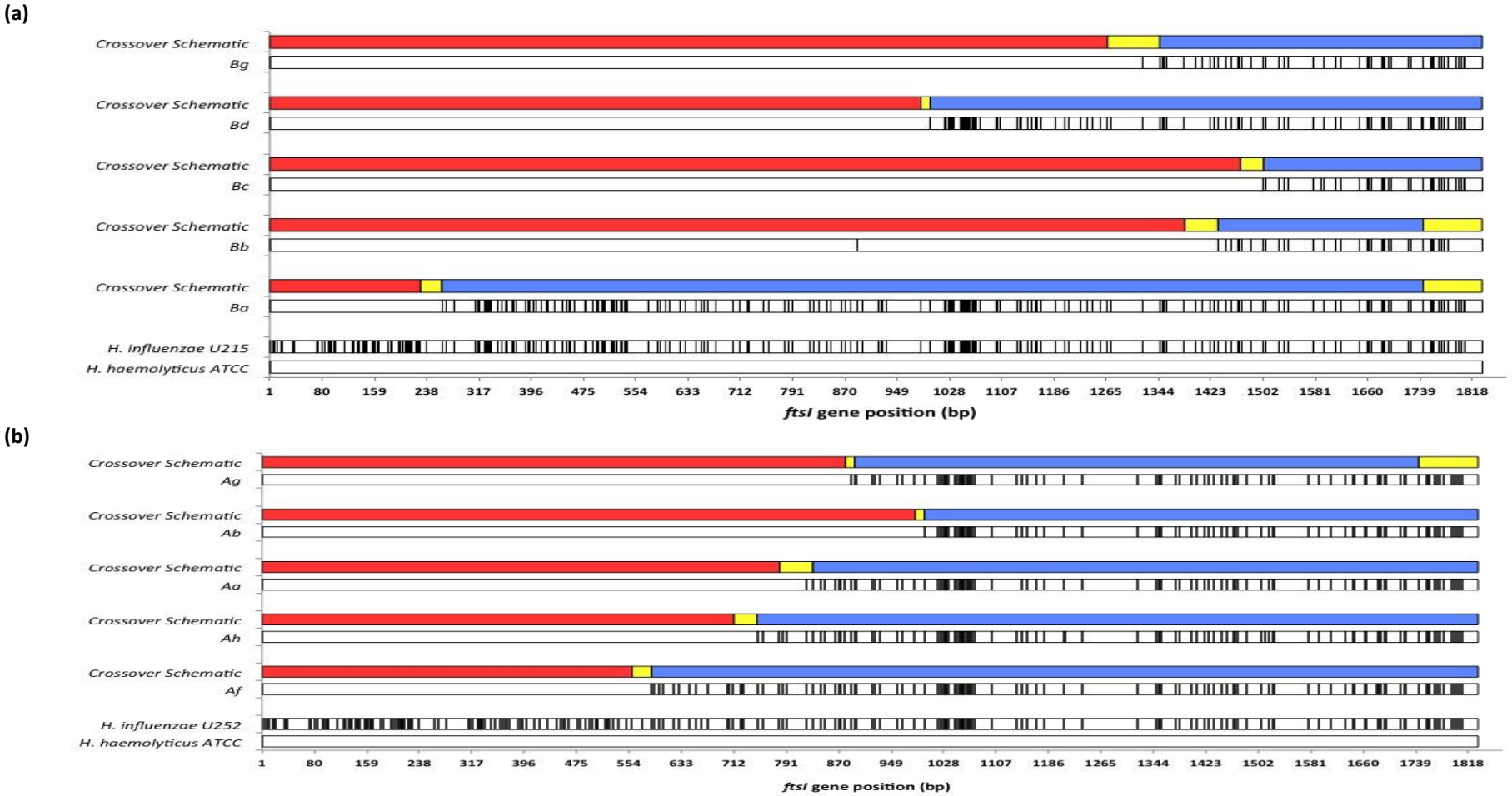


Figure 7.4 Mosaic *ftsI* gene structures identified in the *H. haemolyticus* ATCC 33390 recipient inter-species transformants.
Schematic representations of the divergence in the *ftsI* gene on transformants compared to the *ftsI* sequence of recipient isolate *H. influenzae* Rd. Solid black lines, denote the position of the nucleotides that differ from the corresponding nucleotide in the reference sequence. Crossover schematics show the proportion of the *ftsI* gene most similar to the donor or recipient; Solid red shading, most similar to recipient DNA from *H. haemolyticus* ATCC 33390; Solid yellow shading, crossover location; Solid blue shading, most similar to donor DNA from *H. influenzae* U215 or U252. **(a).** *H. haemolyticus* ATCC/*H. influenzae* U215 transfromants **(b).** *H. haemolyticus* ATCC/*H. influenzae* U252 transformants

7.4.4 Comparison to Clinical Isolates

Comparison of the recombination breakpoints of the inter-species transformants to those identified in the clinical isolates studied by Witherden et al. (2014) (chapter 6), identified the presence of common recombination spots within the *ftsI* gene. In the inter-species transformants where *H. influenzae* Rd was used as the recipient, a number of similarities were identified in the position and size of the inherited recombinant *ftsI* gene fragments. The transformant 7C contained a recombinant fragment similar to that of NTHi Group 3 recombinants identified by Witherden et al. (recombinant fragment 1104-1833 (Gp3); 1104-1758 (7C), whilst transformants 9B and 9D shared similarities with NTHi Group 4 and Group 1 recombinants respectively. Similar findings were identified between the *H. haemolyticus* transformants and the clinical *H. haemolyticus* isolates identified to harbour recombinant *ftsI* genes. Transformants Bg, Ah and Af shared recombination crossover points with the clinical Group 6, 7 and 8 recombinants identified by Witherden et al. (2014) respectively. These findings suggest the presence of common hotspots for recombination of the *ftsI* gene among *H. influenzae* and *H. haemolyticus* species.

The data from the present study has provided some clarification surrounding the dissemination and diversification of BLNAR mediated resistance in both NTHi and *H. haemolyticus* isolates. We have clarified the role of inter-species recombination of the *ftsI* gene in the emergence of BLNAR isolates of *H. influenzae* and *H. haemolyticus*, and confirmed by *in vitro* experiments, the findings of mosaic *ftsI* genes in clinical NTHi and *H. haemolyticus* isolates as discussed in Witherden et al.

(2014) [Chapter 6.0]. We have demonstrated that intra-species recombination of the *ftsI* gene occurs *in vitro*, and this finding supports the notion that both intra-species and inter-species recombination of the *ftsI* gene occurs between *H. influenzae* and *H. haemolyticus* clinical isolates *in vivo*.

Transformants with multiple amino acid substitutions in PBP3 were successfully generated, and this supports the notion that unrelated isolates with the same amino acid substitutions might arise from recombination events, rather than the independent accumulation of identical substitutions, as previously considered. However, as recombination events also resulted in the transfer and formation of different mosaic fragments with varied amino acid substitutions profiles, it is reasonable to suggest that recombination also plays a role in the diversification of amino acid substitution profiles in PBP3.

Admittedly, in this study, we identified the presence of mosaic ftsI gene structures and recombinantion events in the *ftsI* gene through the use of the electroporation techniques, such that it is hard to conclude whether similar events would occur *in vivo* during natural recombination events. However, we can postulate that these *in vitro* experiments represent to some degree physiological events, due to the similarity between recombination hot spots identified in both *in vitro* and *in vivo* experiments conducted throughout this thesis.

Chapter 8.0 – General Discussion

8.1 Summary of Thesis and Major Findings

Various problems with the PCR algorithms routinely used in surveillance studies for the detection of altered PBP3 mediated resistance in *H. influenzae* have been detected during the research projects and publications that comprise this thesis. The major limitations of these PCR approaches stem from the finding that the BLNAR-defining N526K substitution can be encoded by two specific SNPs at position 1576-1578 of *ftsI.* Current PCR primers routinely used in BLNAR detection were originally designed to amplify in the presence of the AAT-AAG SNP encoding N526K, and as a result they fail to amplify N526K encoded by the more recently recognised AAA codon change. This is a significant finding, as these PCR primers are routinely used in surveillance studies to determine the global prevalence of BLNAR isolates. Furthermore, the inability of these primers to detect all N526K genotypes has the potential to significantly underestimate the prevalence of this resistance mechanism on a global scale. In fact, when these primers are used in surveillance studies, up to 50% of all N526K-positive BLNAR isolates may be incorrectly as fully susceptible *H. influenzae* isolates, as they harbour the AAA N526K-positive genotype.

Further limitations to these PCR approaches were identified during the *H. haemolyticus* susceptibility study conducted in Chapter 4. Here a sequence anomaly was identified in *H. haemolyticus* isolates in the forward primer region of PBP3-S

PCR assay. The discovery of this PCR limitation is clinically significant, as these primers are routinely used in respiratory surveillance studies where *H. haemolyticus* isolates are often misidentified as *H. influenzae*, and will result in the mis-categorisation of susceptible *H. haemolyticus* isolates as low-BLNAR isolates of *H. influenzae.* This is a major concern as this PCR assay is routinely used in a wider PCR-algorithm, where PCRs for either OMP *P6* or 16S rRNA genes are used for *H. influenzae* and *H. haemolyticus* differentiation, even though these PCR assays have been shown to be inadequate for the separation of *H. influenzae* and *H. haemolyticus*. As such, it is strongly recommended that the PBP3-S PCR assay should not be used for BLNAR detection in surveillance studies using upper respiratory tract flora where are large proportion of the isolates may actually be *H. haemolyticus*. Instead, we describe the use of a new highly sensitive and specific PCR assay that overcomes the significant limitations of current PCR methodologies used for the detection of altered PBP3 mediated resistance in *H. influenzae* and *H. haemolyticus*.

This thesis also demonstrates for the first time that *H. haemolyticus* isolates harbour the same mechanisms of β-lactam resistance as *H. influenzae.* The phenotypic and genotypic characterisation of normal flora *H. haemolyticus* isolates identified similar prevalence of β-lactamase production and altered PBP3s as routinely reported in *H. influenzae* isolates. This suggests that the co-localisation of *H. haemolyticus* and *H. influenzae* isolates in the upper respiratory tract might represent a significant reservoir for β-lactam resistance determinants in *H. influenzae*, particularly NTHi. Furthermore, specific sequence analysis of the *ftsI* gene and encoded PBP3s identified that many of the BLNAR-associated

substitutions (including D350N, M377I, V547I and N569S) commonly reported in *H. influenzae* appear to form part of the baseline PBP3 sequence in *H. haemolyticus*. These findings suggest that the horizontal transfer of the *ftsI* gene might occur between *H. influenzae* and *H. haemolyticus* isolates *in vivo*.

The most significant findings that arise from the work presented in this thesis stems from the identification of mosaic *ftsI* gene structures in clinical gBLNAR (N526K-positive) isolates of *H. influenzae* and *H. haemolyticus,* that were shown to develop from homologous recombination of the *ftsI* gene *in vivo*. Through detailed bioinformatics analysis of entire *ftsI* genes it was identified that approximately 30% of *H. influenzae* and *H. haemolyticus* gBLNAR isolates harboured mosaic *ftsI* gene structures, which had resulted from inter-species recombination events. This indicates that the horizontal transfer and subsequent recombination of the *ftsI* gene might be more important than previously described. Subsequent *in vitro* transformation experiments identified that the horizontal transfer and recombination of the *ftsI* gene occurs with similar efficiency in both an inter- and intra-species manner among *H. influenzae* and *H. haemolyticus* populations, and also frequently resulted in mosaic gene structures. When one considers this in light of the high rate of inter-species *ftsI* recombination previously identified in clinical isolates, it can be postulated that recombination of the *ftsI* gene is more common than previously described, and potentially a more significant influence than *de novo* point mutation, on the evolution, diversification and dissemination of BLNAR mediated resistance in *Haemophilus* species.

8.2 Limitations of this Thesis and Potential Future Studies

Whilst the interpretation and conclusions drawn from the data presented in this thesis may have been limited by a number of methodological and study design constraints, a number of future study directions have arisen directly from the research findings presented, with both discussed below.

An indirect limitation on the impact of the findings present in this thesis stems from uncertainty surrounding the clinical significance of isolates with the altered PBP3 genotype. At best, a relatively small proportion of these isolates will have MICs to various β-lactams in the non-susceptible or resistant range, however clinical data on the outcome of β-lactam therapy on infections caused by these isolates is currently still lacking. As a result, future studies need to investigate and monitor the therapeutic outcome of commonly prescribed β-lactam antibiotics used to treat infections caused by gBLNAR (or gBLPACR) isolates of *H. influenzae*.

In a similar fashion, the clinical significance of the BLNAR (and BLPACR) genotypes in isolates of *H. haemolyticus* needs to be fully elucidated in light of the recent reports of *H. haemolyticus* being identified as the pathogenic agent in a number of invasive diseases.

In this thesis we characterised the PBP3 profiles of a number of gBLNAS and gBLNAR isolates of *H. influenzae* and *H. haemolyticus*. Although the role of the BLNAR-defining N526K substitution and the high-BLNAR S385T substitution on β-lactam resistance has been clearly defined through transformation and protein modelling experiments, little is currently known about the importance of other BLNAR-

associated substitutions, particularly D350N, M377I, A502T/V, V547I, N569S, which are commonly reported.

Finally I acknowledge that the inclusion of strain typing data obtained through either PFGE or MLST techniques would strengthen the conclusions drawn in chapters 4 and 6, respectively.

8.3 Conclusions

In conclusion, the research findings presented in this thesis improves our current understanding of altered PBP3 mediated resistance in *H. influenzae*, by providing new fundamental knowledge into the dissemination and diversification of this important resistant mechanism in *Haemophilus* species.

This was achieved through:

- The identification of significant limitations in the PCR assays currently used in the detection of altered PBP3 mediated resistance, and the development (and evaluation) of a new PCR assays to overcome these limitations. This is important because of the increasing use of these genetic detection methods in routine surveillance studies.
- An investigation into the β-lactam resistance mechanisms of *H. haemolyticus* that is important because of the increasing recognition of this organism in the diagnostic laboratory, usually as a contaminant but occasionally as a pathogen (a role that is still being elucidated).

- The clarification of the role of *ftsI* gene recombination (both inter- and intra-species) on the dissemination and diversification of altered PBP3 mediated resistance in *H.* influenzae which is fundamental to understanding the evolution of BLNAR mediated resistance on a global scale.

Chapter 9.0 – References

Anderson, R, Wang, X, Briere, EC, Katz, LS, Cohn, AC, Clark, TA, Messonnier, NE, & Mayer, LW (2012) *Haemophilus haemolyticus* isolates causing clinical disease. *J Clin Microbiol, 50*, 2462-2465.

Andrews, JM (2009) BSAC standardized disc susceptibility testing method (Version 8). *J Antimicrob Chemother, 64*, 454-489.

Antibioitc Expert Group, (2010) Therapeutic guidelines: antibiotics. In Antibiotic Expert Group, Version 14. Melbourne: Therapeutic Guidelines Limited.

Bae, S, Lee, J, Kim, E, Lee, S, Yu, J, & Kang, Y (2010) Antimicrobial resistance in *Haemophilus influenzae* respiratory tract isolates in Korea: results of a nationwide acute respiratory infections surveillance. *Antimicrob Agents Chemother, 54*, 65-71.

Barbosa, AR, Giufre, M, Cerquetti, M, & Bajanca-Lavado, MP (2011) Polymorphism in *ftsI* gene and {beta}-lactam susceptibility in Portuguese *Haemophilus influenzae* strains: clonal dissemination of beta-lactamase-positive isolates with decreased susceptibility to amoxicillin/clavulanic acid. *J Antimicrob Chemother, 66*, 788-796.

Barry, AL, Fuchs, PC, & Brown, SD (2001) Identification of beta-lactamase-negative, ampicillin-resistant strains of *Haemophilus influenzae* with four methods and eight media. *Antimicrob Agents Chemother, 45*, 1585-1588.

Binks, MJ, Temple, B, Kirkham, LA, Wiertsema, SP, Dunne, EM, Richmond, PC, Marsh, RL, Leach, AJ, & Smith-Vaughan, HC (2012) Molecular surveillance of true nontypeable *Haemophilus influenzae*: an evaluation of PCR screening assays. *PLoS One, 7*, e34083.

Brunton, JL, Clare, D, Ehrman, N, & Meier, MA (1983) Evolution of antibiotic resistance plasmids in *Neisseria gonorrhoeae* and *Haemophilus* species. *Clin Invest Med, 6*, 221-228.

Cerquetti, M, Ciofi degli Atti, ML, Renna, G, Tozzi, AE, Garlaschi, ML, & Mastrantonio, P (2000) Characterization of non-type B *Haemophilus influenzae* strains isolated from patients with invasive disease. The HI Study Group. *J Clin Microbiol, 38*, 4649-4652.

Chang, A, Adlowitz, DG, Yellamatty, E, & Pichichero, M (2010) *Haemophilus influenzae* outer membrane protein P6 molecular characterization may not differentiate all strains of *H. Influenzae* from *H. haemolyticus*. *J Clin Microbiol, 48*, 3756-3757.

Chen, I, & Dubnau, D (2004) DNA uptake during bacterial transformation. *Nature reviews Microbiology, 2*, 241-249.

Clairoux, N, Picard, M, Brochu, A, Rousseau, N, Gourde, P, Beauchamp, D, Parr, TR, Jr., Bergeron, MG, & Malouin, F (1992) Molecular basis of the non-beta-lactamase-mediated resistance to beta-lactam antibiotics in strains of *Haemophilus influenzae* isolated in Canada. *Antimicrob Agents Chemother, 36*, 1504-1513.

Clinical and Laboratory Standards Institute [CLSI] (2010) Performane Standards for Antimicrobial Susceptibility Testing: Twentieth Informational Supplement *M100-S20*. In CLSI (Ed.). USA: Wayne, PA.

Clinical and Laboratory Standards Institute [CLSI] (2013) Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement: *M100-S23*. In CLSI (Ed.). USA: Wayne, PA.

Connor, TR, Corander, J, & Hanage, WP (2012) Population subdivision and the detection of recombination in non-typable *Haemophilus influenzae*. *Microbiology, 158*, 2958-2964.

Dabernat, H, Delmas, C, Seguy, M, Pelissier, R, Faucon, G, Bennamani, S, & Pasquier, C (2002) Diversity of beta-lactam resistance-conferring amino acid substitutions in penicillin-binding protein 3 of *Haemophilus influenzae*. *Antimicrob Agents Chemother, 46*, 2208-2218.

Doern, GV, Brueggemann, AB, Pierce, G, Holley, HP, Jr., & Rauch, A (1997) Antibiotic resistance among clinical isolates of *Haemophilus influenzae* in the United States in 1994 and 1995 and detection of beta-lactamase-positive strains resistant to amoxicillin-clavulanate: results of a national multicenter surveillance study. *Antimicrob Agents Chemother, 41*, 292-297.

Doern, GV, Jones, RN, Pfaller, MA, & Kugler, K (1999) *Haemophilus influenzae* and *Moraxella catarrhalis* from patients with community-acquired respiratory tract infections: antimicrobial susceptibility patterns from the SENTRY antimicrobial Surveillance Program (United States and Canada, 1997). *Antimicrob Agents Chemother, 43*, 385-389.

Dowson, CG, Hutchison, A, Brannigan, JA, George, RC, Hansman, D, Linares, J, Tomasz, A, Smith, JM, & Spratt, BG (1989) Horizontal transfer of penicillin-binding protein genes in penicillin-resistant clinical isolates of *Streptococcus pneumoniae*. *Proceedings of the National Academy of Sciences of the United States of America, 86*, 8842-8846.

Erwin, AL, Sandstedt, SA, Bonthuis, PJ, Geelhood, JL, Nelson, KL, Unrath, WC, Diggle, MA, Theodore, MJ, Pleatman, CR, Mothershed, EA, Sacchi, CT, Mayer, LW, Gilsdorf, JR, & Smith, AL (2008) Analysis of genetic relatedness of *Haemophilus influenzae* isolates by multilocus sequence typing. *J Bacteriol, 190*, 1473-1483.

European Committee on Antimicrobial Susceptibility Testing [EUCAST] (2013) Breakpoint tables for interpretation of MICs and zone diameters. Version 4.0.

Farrell, DJ, Morrissey, I, Bakker, S, Buckridge, S, & Felmingham, D (2005) Global distribution of TEM-1 and ROB-1 beta-lactamases in *Haemophilus influenzae*. *J Antimicrob Chemother, 56*, 773-776.

Fenger, MG, Ridderberg, W, Olesen, HV, & Norskov-Lauritsen, N (2012) Low occurrence of 'non-haemolytic *Haemophilus haemolyticus*' misidentified as *Haemophilus influenzae* in cystic fibrosis respiratory specimens, and frequent recurrence of persistent *H. influenzae* clones despite antimicrobial treatment. *Int J Med Microbiol, 302*, 315-319.

Fischer, NJ (2014) *Haemophilus influenzae* serotype a septic arthritis in an immunized central Australian indigenous child. *Int J Infect Dis*.

Fleischmann, RD, Adams, MD, White, O, Clayton, RA, Kirkness, EF, Kerlavage, AR, Bult, CJ, Tomb, JF, Dougherty, BA, Merrick, JM, & et al. (1995) Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science, 269*, 496-512.

Fleury, C, Resman, F, Rau, J, & Riesbeck, K (2013) Prevalence, distribution and transfer of small beta-lactamase-containing plasmids in Swedish *Haemophilus influenzae*. *J Antimicrob Chemother*.

Fluit, AC, Florijn, A, Verhoef, J, & Milatovic, D (2005) Susceptibility of European beta-lactamase-positive and -negative *Haemophilus influenzae* isolates from the periods 1997/1998 and 2002/2003. *J Antimicrob Chemother, 56*, 133-138.

Foxwell, AR, Kyd, JM, & Cripps, AW (1998) Nontypeable *Haemophilus influenzae*: pathogenesis and prevention. *Microbiol Molecular Biol Reviews 62*, 294-308.

Fung, WW, O'Dwyer, CA, Sinha, S, Brauer, AL, Murphy, TF, Kroll, JS, & Langford, PR (2006) Presence of copper- and zinc-containing superoxide dismutase in commensal *Haemophilus haemolyticus* isolates can be used as a marker to discriminate them from nontypeable *H. influenzae* isolates. *J Clin Microbiol, 44*, 4222-4226.

Garcia-Cobos, S, Campos, J, Lazaro, E, Roman, F, Cercenado, E, Garcia-Rey, C, Perez-Vazquez, M, Oteo, J, & de Abajo, F (2007) Ampicillin-resistant non-beta-lactamase-producing *Haemophilus influenzae* in Spain: recent emergence of clonal isolates with increased resistance to cefotaxime and cefixime. *Antimicrob Agents Chemother, 51*, 2564-2573.

Garcia-Cobos, S, Campos, J, Cercenado, E, Roman, F, Lazaro, E, Perez-Vazquez, M, de Abajo, F, & Oteo, J (2008a) Antibiotic resistance in *Haemophilus influenzae* decreased, except for beta-lactamase-negative amoxicillin-resistant isolates, in parallel with community antibiotic consumption in Spain from 1997 to 2007. *Antimicrob Agents Chemother, 52*, 2760-2766.

Garcia-Cobos, S, Campos, J, Roman, F, Carrera, C, Perez-Vazquez, M, Aracil, B, & Oteo, J (2008b) Low beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* strains are best detected by testing amoxicillin susceptibility by the broth microdilution method. *Antimicrob Agents Chemother, 52*, 2407-2414.

Garcia-Cobos, S, Arroyo, M, Campos, J, Perez-Vazquez, M, Aracil, B, Cercenado, E, Orden, B, Lara, N, & Oteo, J (2013) Novel mechanisms of resistance to beta-lactam antibiotics in *Haemophilus parainfluenzae*: beta-lactamase-negative ampicillin resistance and inhibitor-resistant TEM beta-lactamases. *J Antimicrob Chemother, 68*, 1054-1059.

Gomes, JP, Bruno, WJ, Nunes, A, Santos, N, Florindo, C, Borrego, MJ, & Dean, D (2007) Evolution of *Chlamydia trachomatis* diversity occurs by widespread interstrain recombination involving hotspots. *Genome Research, 17*, 50-60.

Gotoh, K, Qin, L, Watanabe, K, Anh, DD, Huong Ple, T, Anh, NT, Cat, ND, Ha, LL, Ai le, TT, Tien, NM, Minh, TT, Oishi, K, & Watanabe, H (2008) Prevalence of *Haemophilus influenzae* with resistant genes isolated from young children with acute lower respiratory tract infections in Nha Trang, Vietnam. *J Infect Chemother, 14*, 349-353.

Hakenbeck, R (1998) Mosaic genes and their role in penicillin-resistant *Streptococcus pneumoniae*. *Electrophoresis, 19*, 597-601.

Hakenbeck, R, Balmelle, N, Weber, B, Gardes, C, Keck, W, & de Saizieu, A (2001) Mosaic genes and mosaic chromosomes: intra- and interspecies genomic variation of *Streptococcus pneumoniae*. *Infect Immun, 69*, 2477-2486.

Hare, KM, Binks, MJ, Grimwood, K, Chang, AB, Leach, AJ, & Smith-Vaughan, H (2012) Culture and PCR Detection of *Haemophilus influenzae* and *Haemophilus haemolyticus* in Australian Indigenous Children with Bronchiectasis. *J Clin Microbiol, 50*, 2444-2445.

Hasegawa, K, Yamamoto, K, Chiba, N, Kobayashi, R, Nagai, K, Jacobs, MR, Appelbaum, PC, Sunakawa, K, & Ubukata, K (2003) Diversity of ampicillin-resistance genes in *Haemophilus influenzae* in Japan and the United States. *Microb Drug Resist, 9*, 39-46.

Hasegawa, K, Chiba, N, Kobayashi, R, Murayama, SY, Iwata, S, Sunakawa, K, & Ubukata, K (2004) Rapidly increasing prevalence of beta-lactamase-nonproducing, ampicillin-resistant *Haemophilus influenzae* type b in patients with meningitis. *Antimicrob Agents Chemother, 48*, 1509-1514.

Hasegawa, K, Kobayashi, R, Takada, E, Ono, A, Chiba, N, Morozumi, M, Iwata, S, Sunakawa, K, Ubukata, K, & Nationwide Surveillance for Bacterial, M (2006) High prevalence of type b beta-lactamase-non-producing ampicillin-resistant *Haemophilus influenzae* in meningitis: the situation in Japan where Hib vaccine has not been introduced. *J Antimicrob Chemother, 57*, 1077-1082.

Heelan, JS, Chesney, D, & Guadagno, G (1992) Investigation of ampicillin-intermediate strains of *Haemophilus influenzae* by using the disk diffusion procedure and current National Committee for Clinical Laboratory Standards guidelines. *J Clin Microbiol, 30*, 1674-1677.

Herbert, MA, Hood, DW, & Richard Maxon, E (2003) *Haemophilus influenzae* Protocols. In D. W. H. a. E. R. M. Mark A. Herbet (Ed.), *Methods in Molecular Medicine*. Totowa, New Jersey: Humana Press.

Hoban, D, & Felmingham, D (2002) The PROTEKT surveillance study: antimicrobial susceptibility of *Haemophilus influenzae* and *Moraxella catarrhalis* from community-acquired respiratory tract infections. *J Antimicrob Chemother, 50 Suppl S1*, 49-59.

Hoberman, A, Marchant, CD, Kaplan, SL, & Feldman, S (2002) Treatment of acute otitis media consensus recommendations. *Clin Pediatr (Phila), 41*, 373-390.

Hotomi, M, Fujihara, K, Sakai, A, Billal, DS, Shimada, J, Suzumoto, M, & Yamanaka, N (2006) Antimicrobial resistance of *Haemophilus influenzae* isolated from the nasopharynx of Japanese children with acute otitis media. *Acta Otolaryngol, 126*, 240-247.

Hotomi, M, Fujihara, K, Billal, DS, Suzuki, K, Nishimura, T, Baba, S, & Yamanaka, N (2007) Genetic characteristics and clonal dissemination of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* strains isolated from the upper respiratory tract of patients in Japan. *Antimicrob Agents Chemother, 51*, 3969-3976.

Huang, MM, Arnheim, N, & Goodman, MF (1992) Extension of base mispairs by Taq DNA polymerase: implications for single nucleotide discrimination in PCR. *Nucleic Acids Res, 20*, 4567-4573.

James, PA, Lewis, DA, Jordens, JZ, Cribb, J, Dawson, SJ, & Murray, SA (1996) The incidence and epidemiology of beta-lactam resistance in *Haemophilus influenzae*. *J Antimicrob Chemother, 37*, 737-746.

Jansen, WT, Verel, A, Beitsma, M, Verhoef, J, & Milatovic, D (2006) Longitudinal European surveillance study of antibiotic resistance of *Haemophilus influenzae*. *J Antimicrob Chemother, 58*, 873-877.

Jansen, WT, Verel, A, Beitsma, M, Verhoef, J, & Milatovic, D (2008) Surveillance study of the susceptibility of *Haemophilus influenzae* to various antibacterial agents in Europe and Canada. *Curr Med Res Opin, 24*, 2853-2861.

Jordan, IK, Conley, AB, Antonov, IV, Arthur, RA, Cook, ED, Cooper, GP, Jones, BL, Knipe, KM, Lee, KJ, Liu, X, Mitchell, GJ, Pande, PR, Petit, RA, Qin, S, Rajan, VN, Sarda, S, Sebastian, A, Tang, S, Thapliyal, R, Varghese, NJ, Ye, T, Katz, LS, Wang, X, Rowe, L, Frace, M, & Mayer, LW (2011) Genome sequences for five strains of the emerging pathogen *Haemophilus haemolyticus*. *J Bacteriol, 193*, 5879-5880.

Kaczmarek, FS, Gootz, TD, Dib-Hajj, F, Shang, W, Hallowell, S, & Cronan, M (2004) Genetic and molecular characterization of beta-lactamase-negative ampicillin-resistant Haemophilus influenzae with unusually high resistance to ampicillin. *Antimicrob Agents Chemother, 48*, 1630-1639.

Karlowsky, JA, Verma, G, Zhanel, GG, & Hoban, DJ (2000) Presence of ROB-1 beta-lactamase correlates with cefaclor resistance among recent isolates of *Haemophilus influenzae*. *J Antimicrob Chemother, 45*, 871-875.

Karlowsky, JA, Critchley, IA, Blosser-Middleton, RS, Karginova, EA, Jones, ME, Thornsberry, C, & Sahm, DF (2002) Antimicrobial surveillance of *Haemophilus influenzae* in the United States during 2000-2001 leads to detection of clonal dissemination of a beta-lactamase-negative and ampicillin-resistant strain. *J Clin Microbiol, 40*, 1063-1066.

Karpanoja, P, Nissinen, A, Huovinen, P, & Sarkkinen, H (2004) Disc diffusion susceptibility testing of *Haemophilus influenzae* by NCCLS methodology using low-strength ampicillin and co-amoxiclav discs. *J Antimicrob Chemother, 53*, 660-663.

Kilian, M (1976) A taxonomic study of the genus *Haemophilus*, with the proposal of a new species. *J Gen Microbiol, 93*, 9-62.

Kilian, M (2003) *Haemophilus*. In P. R. Murray Ed, *Manual of Clinical Microbiology* (Vol. 1, pp. 623-635). Washington, DC: ASM PRESS.

Kim, IS, Ki, CS, Kim, S, Oh, WS, Peck, KR, Song, JH, Lee, K, & Lee, NY (2007) Diversity of ampicillin resistance genes and antimicrobial susceptibility patterns in *Haemophilus influenzae* strains isolated in Korea. *Antimicrob Agents Chemother, 51*, 453-460.

Kimura, M (1980) A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol, 16*, 111-120.

King, P (2012) *Haemophilus influenzae* and the lung (*Haemophilus* and the lung). *Clin Transl Med, 1*, 10.

Kirkham, LA, Wiertsema, SP, Mowe, EN, Bowman, JM, Riley, TV, & Richmond, PC (2010) Nasopharyngeal carriage of *Haemophilus haemolyticus* in otitis-prone and healthy children. *J Clin Microbiol, 48*, 2557-2559.

Kishii, K, Chiba, N, Morozumi, M, Hamano-Hasegawa, K, Kurokawa, I, Masaki, J, & Ubukata, K (2010) Diverse mutations in the *ftsI* gene in ampicillin-resistant

Haemophilus influenzae isolates from pediatric patients with acute otitis media. *J Infect Chemother, 16*, 87-93.

Kishii, K, Morozumi, M, Chiba, N, Ono, A, & Ubukata, K (2011) Direct detection by real-time PCR of *ftsI* gene mutations affecting MICs of beta-lactam agents for *Haemophilus influenzae* isolates from meningitis. *J Infect Chemother*.

Krieg, NRL, Wolfgang; Whitman, William B.; Hedlund, Brian P.; Paster, Bruce J.; Staley, James T.; Ward, Naomi; Brown, Daniel (2001) *Bergey's manual of Systematic Bacteriology* (2nd ed.). New York: Springer.

Kroll, JS, Langford, PR, Wilks, KE, & Keil, AD (1995) Bacterial [Cu,Zn]-superoxide dismutase: phylogenetically distinct from the eukaryotic enzyme, and not so rare after all! *Microbiology, 141 (Pt 9)*, 2271-2279.

Kubota, T, Higa, F, Kusano, N, Nakasone, I, Haranage, S, Tateyama, M, Yamane, N, & Fujita, J (2006) Genetic analyses of beta-lactamase negative ampicillin-resistant strains of *Haemophilus influenzae* isolated in Okinawa, Japan. *Jpn J Infect Dis, 59*, 36-41.

Kuhnert, P, & Christensen, H (2008) *Pasteurellaceae*: Biology, Genomics and Molecular Aspects. Christensen (Ed.). Norfolk, UK: Caister Academic Press.

Leaves, NI, Dimopoulou, I, Hayes, I, Kerridge, S, Falla, T, Secka, O, Adegbola, RA, Slack, MP, Peto, TE, & Crook, DW (2000) Epidemiological studies of large resistance plasmids in *Haemophilus*. *J Antimicrob Chemother, 45*, 599-604.

Livermore, DM, & Brown, DF (2001) Detection of beta-lactamase-mediated resistance. *J Antimicrob Chemother, 48 Suppl 1*, 59-64.

Lole, KS, Bollinger, RC, Paranjape, RS, Gadkari, D, Kulkarni, SS, Novak, NG, Ingersoll, R, Sheppard, HW, & Ray, SC (1999) Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *Journal of virology, 73*, 152-160.

MacNeil, JR, Cohn, AC, Farley, M, Mair, R, Baumbach, J, Bennett, N, Gershman, K, Harrison, LH, Lynfield, R, Petit, S, Reingold, A, Schaffner, W, Thomas, A, Coronado, F, Zell, ER, Mayer, LW, Clark, TA, & Messonnier, NE (2011) Current epidemiology and trends in invasive *Haemophilus influenzae* disease--United States, 1989-2008. *Clin Infect Dis, 53*, 1230-1236.

Maiden, MC (1998) Horizontal genetic exchange, evolution, and spread of antibiotic resistance in bacteria. *Clin Infect Dis, 27 Suppl 1*, S12-20.

Marco, F, Garcia-de-Lomas, J, Garcia-Rey, C, Bouza, E, Aguilar, L, Fernandez-Mazarrasa, C, & Spanish Surveillance Group for Respiratory, P (2001) Antimicrobial susceptibilities of 1,730 *Haemophilus influenzae* respiratory tract isolates in Spain in 1998-1999. *Antimicrob Agents Chemother, 45*, 3226-3228.

Markowitz, SM (1980) Isolation of an ampicillin-resistant, non-beta-lactamase-producing strain of *Haemophilus influenzae*. *Antimicrob Agents Chemother, 17*, 80-83.

Matic, V, Bozdogan, B, Jacobs, MR, Ubukata, K, & Appelbaum, PC (2003) Contribution of beta-lactamase and PBP amino acid substitutions to amoxicillin/clavulanate resistance in beta-lactamase-positive, amoxicillin/clavulanate-resistant *Haemophilus influenzae*. *J Antimicrob Chemother, 52*, 1018-1021.

McCrea, KW, Xie, J, LaCross, N, Patel, M, Mukundan, D, Murphy, TF, Marrs, CF, & Gilsdorf, JR (2008) Relationships of nontypeable *Haemophilus influenzae* strains to hemolytic and nonhemolytic *Haemophilus haemolyticus* strains. *J Clin Microbiol, 46*, 406-416.

McIntyre, PB, O'Brien, KL, Greenwood, B, & van de Beek, D (2012) Effect of vaccines on bacterial meningitis worldwide. *Lancet, 380*, 1703-1711.

Meats, E, Feil, EJ, Stringer, S, Cody, AJ, Goldstein, R, Kroll, JS, Popovic, T, & Spratt, BG (2003) Characterization of encapsulated and noncapsulated *Haemophilus influenzae* and determination of phylogenetic relationships by multilocus sequence typing. *J Clin Microbiol, 41*, 1623-1636.

Medeiros, AA, & O'Brien, TF (1975) Ampicillin-resistant *Haemophilus influenzae* type B possessing a TEM-type beta-lactamase but little permeability barrier to ampicillin. *Lancet, 1*, 716-719.

Mendelman, PM, Chaffin, DO, Stull, TL, Rubens, CE, Mack, KD, & Smith, AL (1984) Characterization of non-beta-lactamase-mediated ampicillin resistance in *Haemophilus influenzae*. *Antimicrob Agents Chemother, 26*, 235-244.

Mendelman, PM, Chaffin, DO, Clausen, C, Stull, TL, Needham, C, Williams, JD, & Smith, AL (1986) Failure to detect ampicillin-resistant, non-beta-lactamase-producing *Haemophilus influenzae* by standard disk susceptibility testing. *Antimicrob Agents Chemother, 30*, 274-280.

Mendelman, PM, Chaffin, DO, Krilov, LR, Kalaitzoglou, G, Serfass, DA, Onay, O, Wiley, EA, Overturf, GD, & Rubin, LG (1990a) Cefuroxime treatment failure of nontypable *Haemophilus influenzae* meningitis associated with alteration of penicillin-binding proteins. *J Infect Dis, 162*, 1118-1123.

Mendelman, PM, Chaffin, DO, & Kalaitzoglou, G (1990b) Penicillin-binding proteins and ampicillin resistance in *Haemophilus influenzae*. *J Antimicrob Chemother, 25*, 525-534.

Molina, JM, Cordoba, J, Monsoliu, A, Diosdado, N, & Gobernado, M (2003) *Haemophilus influenzae* and betalactam resistance: description of bla TEM gene deletion. *Rev Esp Quimioter, 16*, 195-203.

Mukundan, D, Ecevit, Z, Patel, M, Marrs, CF, & Gilsdorf, JR (2007) Pharyngeal colonization dynamics of *Haemophilus influenzae* and *Haemophilus haemolyticus* in healthy adult carriers. *J Clin Microbiol, 45*, 3207-3217.

Murphy, TF (2003) Respiratory infections caused by non-typeable *Haemophilus influenzae*. *Curr Opin Infect Dis, 16*, 129-134.

Murphy, TF, Brauer, AL, Sethi, S, Kilian, M, Cai, X, & Lesse, AJ (2007) *Haemophilus haemolyticus*: a human respiratory tract commensal to be distinguished from *Haemophilus influenzae*. *J Infect Dis, 195*, 81-89.

Murphy, TF, Faden, H, Bakaletz, LO, Kyd, JM, Forsgren, A, Campos, J, Virji, M, & Pelton, SI (2009) Nontypeable *Haemophilus influenzae* as a pathogen in children. *Pediatr Infect Dis J, 28*, 43-48.

Musser, JM, Barenkamp, SJ, Granoff, DM, & Selander, RK (1986) Genetic relationships of serologically nontypable and serotype b strains of *Haemophilus influenzae*. *Infect Immun, 52*, 183-191.

Nakamura, S, Yanagihara, K, Morinaga, Y, Izumikawa, K, Seki, M, Kakeya, H, Yamamoto, Y, Kamihira, S, & Kohno, S (2009) Multiplex real-time polymerase

chain reaction for rapid detection of beta-lactamase-negative, ampicillin-resistant *Haemophilus influenzae*. *Diagn Microbiol Infect Dis, 64*, 64-69.

Nei, MaK, S. (2000) *Molecular Evolution and Phylogenetics*. New York: Oxford University Press.

Niki, Y, Hanaki, H, Matsumoto, T, Yagisawa, M, Kohno, S, Aoki, N, Watanabe, A, Sato, J, Hattori, R, Koashi, N, Terada, M, Kozuki, T, Maruo, A, Morita, K, Ogasawara, K, Takahashi, Y, Matsuda, K, Nakanishi, K, Sunakawa, K, Takeuchi, K, Fujimura, S, Takeda, H, Ikeda, H, Sato, N, Niitsuma, K, Saito, M, Koshiba, S, Kaneko, M, Miki, M, Nakanowatari, S, Takahashi, H, Utagawa, M, Nishiya, H, Kawakami, S, Aoki, Y, Chonabayashi, N, Sugiura, H, Ichioka, M, Goto, H, Kurai, D, Saraya, T, Okazaki, M, Yoshida, K, Yoshida, T, Tsukada, H, Imai, Y, Honma, Y, Yamamoto, T, Kawai, A, Mikamo, H, Takesue, Y, Wada, Y, Miyara, T, Toda, H, Mitsuno, N, Fujikawa, Y, Nakajima, H, Kubo, S, Ohta, Y, Mikasa, K, Kasahara, K, Koizumi, A, Sano, R, Yagi, S, Takaya, M, Kurokawa, Y, Kusano, N, Mihara, E, Nose, M, Kuwabara, M, Fujiue, Y, Ishimaru, T, Matsubara, N, Kawasaki, Y, Tokuyasu, H, Masui, K, Kido, M, Ota, T, Honda, J, Kadota, J, Hiramatsu, K, Aoki, Y, Nagasawa, Z, Yanagihara, K, Fujita, J, Tateyama, M, & Totsuka, K (2011) Nationwide surveillance of bacterial respiratory pathogens conducted by the Japanese Society of Chemotherapy in 2008: general view of the pathogens' antibacterial susceptibility. *J Infect Chemother, 17*, 510-523.

Norskov-Lauritsen, N, Bruun, B, & Kilian, M (2005) Multilocus sequence phylogenetic study of the genus *Haemophilus* with description of *Haemophilus pittmaniae* sp. nov. *International journal of systematic and evolutionary microbiology, 55*, 449-456.

Norskov-Lauritsen, N (2009) Detection of cryptic genospecies misidentified as *Haemophilus influenzae* in routine clinical samples by assessment of marker genes *fucK*, *hap*, and *sodC*. *J Clin Microbiol, 47*, 2590-2592.

Norskov-Lauritsen, N, Overballe, MD, & Kilian, M (2009) Delineation of the species *Haemophilus influenzae* by phenotype, multilocus sequence phylogeny, and detection of marker genes. *J Bacteriol, 191*, 822-831.

Norskov-Lauritsen, N, Ridderberg, W, Erikstrup, LT, & Fuursted, K (2011) Evaluation of disk diffusion methods to detect low-level beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *Apmis, 119*, 385-392.

Norskov-Lauritsen, N (2011) Increased level of intragenomic 16S rRNA gene heterogeneity in commensal strains closely related to *Haemophilus influenzae*. *Microbiology, 157*, 1050-1055.

Ohkoshi, Y, Yokota, S, Sato, K, Hayashi, T, Matsuda, K, Kuwahara, O, Akizawa, H, & Fujii, N (2008) Antibiotic susceptibility of *Haemophilus influenzae* strains isolated from various clinical sources in Hokkaido Prefecture, Japan. *J Infect Chemother, 14*, 93-98.

Osaki, Y, Sanbongi, Y, Ishikawa, M, Kataoka, H, Suzuki, T, Maeda, K, & Ida, T (2005) Genetic approach to study the relationship between penicillin-binding protein 3 mutations and *Haemophilus influenzae* beta-lactam resistance by using site-directed mutagenesis and gene recombinants. *Antimicrob Agents Chemother, 49*, 2834-2839.

Pares, S, Mouz, N, Petillot, Y, Hakenbeck, R, & Dideberg, O (1996) X-ray structure of *Streptococcus pneumoniae* PBP2x, a primary penicillin target enzyme. *Nat Struct Biol, 3*, 284-289.

Park, C, Kim, KH, Shin, NY, Byun, JH, Kwon, EY, Lee, JW, Kwon, HJ, Choi, EY, Lee, DG, Sohn, WY, & Kang, JH (2013) Genetic diversity of the *ftsI* gene in beta-lactamase-nonproducing ampicillin-resistant and beta-lactamase-producing amoxicillin-clavulanic acid-resistant nasopharyngeal *Haemophilus influenzae* strains isolated from children in South Korea. *Microb Drug Resist, 19*, 224-230.

Parr, TR, Jr., & Bryan, LE (1984) Mechanism of resistance of an ampicillin-resistant, beta-lactamase-negative clinical isolate of *Haemophilus influenzae* type b to beta-lactam antibiotics. *Antimicrob Agents Chemother, 25*, 747-753.

Peltola, H (2000) Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21st century: global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates. *Clin Microbiol Rev, 13*, 302-317.

Pittman, M (1931) Variation and Type Specificity in the Bacterial Species *Hemophilus Influenzae*. *J Exp Med, 53*, 471-492.

Qin, L, Watanabe, H, Asoh, N, Watanabe, K, Oishi, K, Mizota, T, & Nagatake, T (2007) Short report: antimicrobial susceptibility and genetic characteristics of *Haemophilus influenzae* isolated from patients with respiratory tract infections between 1987 and 2000, including beta-lactamase-negative ampicillin-resistant strains. *Epidemiol Infect, 135*, 665-668.

Robertson, DL, Hahn, BH, & Sharp, PM (1995) Recombination in AIDS viruses. *J Mol Evol, 40*, 249-259.

Robson, J, Collignon, Peter, Pearson, Julie, and Bell, Jan. (2006) *Haemophilus influenzae* Survey 2006 - Antimicrobial Susceptibility Report. In: The Australian Group on Antimicrobial Resistance.

Rubin, LG, Medeiros, AA, Yolken, RH, & Moxon, ER (1981) Ampicillin treatment failure of apparently beta-lactamase-negative *Haemophilus influenzae* type b meningitis due to novel beta-lactamase. *Lancet, 2*, 1008-1010.

Saitou, N, & Nei, M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular biology and evolution, 4*, 406-425.

Sakai, A, Hotomi, M, Billal, DS, Yamauchi, K, Shimada, J, Tamura, S, Fujihara, K, & Yamanaka, N (2005) Evaluation of mutations in penicillin binding protein-3 gene of non-typeable *Haemophilus influenzae* isolated from the nasopharynx of children with acute otitis media. *Acta Otolaryngol, 125*, 180-183.

Salminen, MO, Carr, JK, Burke, DS, & McCutchan, FE (1995) Identification of breakpoints in intergenotypic recombinants of HIV type 1 by bootscanning. *AIDS research and human retroviruses, 11*, 1423-1425.

San Millan, A, Giufre, M, Escudero, JA, Hidalgo, L, Gutierrez, B, Cerquetti, M, & Gonzalez-Zorn, B (2011) Contribution of ROB-1 and PBP3 mutations to the resistance phenotype of a beta-lactamase-positive amoxicillin/clavulanic acid-resistant *Haemophilus influenzae* carrying plasmid pB1000 in Italy. *J Antimicrob Chemother, 66*, 96-99.

Sanbongi, Y, Suzuki, T, Osaki, Y, Senju, N, Ida, T, & Ubukata, K (2006) Molecular evolution of beta-lactam-resistant *Haemophilus influenzae*: 9-year surveillance of penicillin-binding protein 3 mutations in isolates from Japan. *Antimicrob Agents Chemother, 50*, 2487-2492.

Sandstedt, SA, Zhang, L, Patel, M, McCrea, KW, Qin, Z, Marrs, CF, & Gilsdorf, JR (2008) Comparison of laboratory-based and phylogenetic methods to distinguish between *Haemophilus influenzae* and *H. haemolyticus*. *J Microbiol Methods, 75*, 369-371.

Scriver, SR, Walmsley, SL, Kau, CL, Hoban, DJ, Brunton, J, McGeer, A, Moore, TC, & Witwicki, E (1994a) Determination of antimicrobial susceptibilities of Canadian isolates of *Haemophilus influenzae* and characterization of their beta-lactamases. Canadian *Haemophilus* Study Group. *Antimicrob Agents Chemother, 38*, 1678-1680.

Scriver, SR, Hoban, DJ, McGeer, A, Moore, TC, Walmsley, SL, & Low, DE (1994b) Surveillance of susceptibility testing methodologies for *Haemophilus influenzae* in Canada, including evaluation of disk diffusion test. *J Clin Microbiol, 32*, 2013-2015.

Shuel, ML, & Tsang, RS (2009) Canadian beta-lactamase-negative *Haemophilus influenzae* isolates showing decreased susceptibility toward ampicillin have significant penicillin binding protein 3 mutations. *Diagn Microbiol Infect Dis, 63*, 379-383.

Shuel, ML, Karlowsky, KE, Law, DK, & Tsang, RS (2011) Nonencapsulated or nontypeable *Haemophilus influenzae* are more likely than their encapsulated or serotypeable counterparts to have mutations in their fucose operon. *Can J Microbiol, 57*, 982-986.

Sibold, C, Wang, J, Henrichsen, J, & Hakenbeck, R (1992) Genetic relationships of penicillin-susceptible and -resistant *Streptococcus pneumoniae* strains isolated on different continents. *Infect Immun, 60*, 4119-4126.

Skaare, D, Allum, AG, Anthonisen, IL, Jenkins, A, Lia, A, Strand, L, Tveten, Y, & Kristiansen, BE (2010) Mutant *ftsI* genes in the emergence of penicillin-binding protein-mediated beta-lactam resistance in *Haemophilus influenzae* in Norway. *Clin Microbiol Infect, 16*, 1117-1124.

Slack, MP, Azzopardi, HJ, Hargreaves, RM, & Ramsay, ME (1998) Enhanced surveillance of invasive *Haemophilus influenzae* disease in England, 1990 to 1996: impact of conjugate vaccines. *Pediatr Infect Dis J, 17*, S204-207.

Smith-Vaughan, HC, Sriprakash, KS, Mathews, JD, & Kemp, DJ (1997) Nonencapsulated *Haemophilus influenzae* in Aboriginal infants with otitis media: prolonged carriage of P2 porin variants and evidence for horizontal P2 gene transfer. *Infect Immun, 65*, 1468-1474.

Smith-Vaughan, HC, Sriprakash, KS, Leach, AJ, Mathews, JD, & Kemp, DJ (1998) Low genetic diversity of H*aemophilus influenzae* type b compared to nonencapsulated *H. influenzae* in a population in which *H. influenzae* is highly endemic. *Infect Immun, 66*, 3403-3409.

Sondergaard, A, Petersen, MT, Fuursted, K, & Norskov-Lauritsen, N (2012) Detection of N526K-substituted penicillin-binding protein 3 conferring low-level mutational resistance to beta-lactam antibiotics in *Haemophilus influenzae*

by disc diffusion testing on Mueller-Hinton agar according to EUCAST guidelines. *J Antimicrob Chemother, 67*, 1401-1404.

Spratt, BG, Bowler, LD, Zhang, QY, Zhou, J, & Smith, JM (1992) Role of interspecies transfer of chromosomal genes in the evolution of penicillin resistance in pathogenic and commensal *Neisseria* species. *J Mol Evol, 34*, 115-125.

Straker, K, Wootton, M, Simm, AM, Bennett, PM, MacGowan, AP, & Walsh, TR (2003) Cefuroxime resistance in non-beta-lactamase *Haemophilus influenzae* is linked to mutations in *ftsI*. *J Antimicrob Chemother, 51*, 523-530.

Takahata, S, Ida, T, Senju, N, Sanbongi, Y, Miyata, A, Maebashi, K, & Hoshiko, S (2007) Horizontal gene transfer of *ftsI*, encoding penicillin-binding protein 3, in *Haemophilus influenzae*. *Antimicrob Agents Chemother, 51*, 1589-1595.

Theodore, MJ, Anderson, RD, Wang, X, Katz, LS, Vuong, JT, Bell, ME, Juni, BA, Lowther, SA, Lynfield, R, MacNeil, JR, & Mayer, LW (2012) Evaluation of new biomarker genes for differentiating *Haemophilus influenzae* from *Haemophilus haemolyticus*. *J Clin Microbiol, 50*, 1422-1424.

Thornsberry, C, & Kirven, LA (1974) Ampicillin resistance in *Haemophilus influenzae* as determined by a rapid test for beta-lactamase production. *Antimicrob Agents Chemother, 6*, 653-654.

Tremblay, LD, L'Ecuyer, J, Provencher, P, & Bergeron, MG (1990) Susceptibility of *Haemophilus influenzae* to antimicrobial agents used in Canada. Canadian Study Group. *Cmaj, 143*, 895-901.

Tristram, S, Jacobs, MR, & Appelbaum, PC (2007) Antimicrobial resistance in *Haemophilus influenzae*. *Clin Microbiol Rev, 20*, 368-389.

Tristram, SG, & Nichols, S (2006) A multiplex PCR for beta-lactamase genes of *Haemophilus influenzae* and description of a new blaTEM promoter variant. *J Antimicrob Chemother, 58*, 183-185.

Tristram, SG, Littlejohn, R, & Bradbury, RS (2010) blaROB-1 presence on pB1000 in *Haemophilus influenzae* is widespread, and variable cefaclor resistance is associated with altered penicillin-binding proteins. *Antimicrob Agents Chemother, 54*, 4945-4947.

Tristram, SG, Franks, LR, & Harvey, GL (2012) Sequences of small blaTEM-encoding plasmids in *Haemophilus influenzae* and description of variants falsely negative for blaTEM by PCR. *J Antimicrob Chemother, 67*, 2621-2625.

Turnidge, J, & Paterson, DL (2007) Setting and revising antibacterial susceptibility breakpoints. *Clin Microbiol Rev, 20*, 391-408.

Ubukata, K, Shibasaki, Y, Yamamoto, K, Chiba, N, Hasegawa, K, Takeuchi, Y, Sunakawa, K, Inoue, M, & Konno, M (2001) Association of amino acid substitutions in penicillin-binding protein 3 with beta-lactam resistance in beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae*. *Antimicrob Agents Chemother, 45*, 1693-1699.

Ubukata, K, Chiba, N, Morozumi, M, Iwata, S, Sunakawa, K, & Working Group of Nationwide Surveillance for Bacterial, M (2013) Longitudinal surveillance of *Haemophilus influenzae* isolates from pediatric patients with meningitis throughout Japan, 2000-2011. *J Infect Chemother, 19*, 34-41.

van der Ploeg, V, Gelinck, LB, Jonkers, IJ, Mourer, JS, & van Dam, AP (2008) Treatment failure in *Haemophilus influenzae* due to a beta-lactamase low-level ampicillin-resistant organism. *J Infect, 57*, 485-488.

Vitovski, S, Dunkin, KT, Howard, AJ, & Sayers, JR (2002) Nontypeable *Haemophilus influenzae* in carriage and disease: a difference in IgA1 protease activity levels. *JAMA : the journal of the American Medical Association, 287*, 1699-1705.

Vu, HT, Yoshida, LM, Suzuki, M, Nguyen, HA, Nguyen, CD, Nguyen, AT, Oishi, K, Yamamoto, T, Watanabe, K, & Vu, TD (2011) Association between nasopharyngeal load of *Streptococcus pneumoniae*, viral coinfection, and radiologically confirmed pneumonia in Vietnamese children. *Pediatr Infect Dis J, 30*, 11-18.

Wang, X, Mair, R, Hatcher, C, Theodore, MJ, Edmond, K, Wu, HM, Harcourt, BH, Carvalho Mda, G, Pimenta, F, Nymadawa, P, Altantsetseg, D, Kirsch, M, Satola, SW, Cohn, A, Messonnier, NE, & Mayer, LW (2011) Detection of bacterial pathogens in Mongolia meningitis surveillance with a new real-time PCR assay to detect *Haemophilus influenzae*. *Int J Med Microbiol, 301*, 303-309.

Watanabe, H, Hoshino, K, Sugita, R, Asoh, N, Watanabe, K, Oishi, K, & Nagatake, T (2004) Possible high rate of transmission of nontypeable *Haemophilus influenzae*, including beta-lactamase-negative ampicillin-resistant strains, between children and their parents. *J Clin Microbiol, 42*, 362-365.

Watt, JP, Wolfson, LJ, O'Brien, KL, Henkle, E, Deloria-Knoll, M, McCall, N, Lee, E, Levine, OS, Hajjeh, R, Mulholland, K, & Cherian, T (2009) Burden of disease caused by Haemophilus influenzae type b in children younger than 5 years: global estimates. *Lancet, 374*, 903-911.

Wiertsema, SP, Kirkham, LA, Corscadden, KJ, Mowe, EN, Bowman, JM, Jacoby, P, Francis, R, Vijayasekaran, S, Coates, HL, Riley, TV, & Richmond, P (2011) Predominance of nontypeable *Haemophilus influenzae* in children with otitis media following introduction of a 3+0 pneumococcal conjugate vaccine schedule. *Vaccine, 29*, 5163-5170.

Winn, W, Allen, S., Janda, W., Koneman, E., Procop, G., Schreckengerer, P., and Woods, G. (2006) *Koneman's Colour Atlas and Textbook of Diagnostic Microbiology*: Lippincott, Williams and Wilkins.

Witherden, EA, Montgomery, J, Henderson, B, & Tristram, SG (2011) Prevalence and genotypic characteristics of beta-lactamase-negative ampicillin-resistant *Haemophilus influenzae* in Australia. *J Antimicrob Chemother, 66*, 1013-1015.

Witherden, EA, Kunde, D, & Tristram, SG (2012) An evaluation of SNP-based PCR methods for the detection of beta-lactamase-negative ampicillin-resistant Haemophilus influenzae. *J Infect Chemother, 18*, 451-455.

Witherden, EA, & Tristram, SG (2013) Prevalence and mechanisms of beta-lactam resistance in *Haemophilus haemolyticus*. *J Antimicrob Chemother, 68*, 1049-1053.

Witherden, EA, Kunde, D, & Tristram, SG (2013) PCR screening for the N526K substitution in isolates of *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother, 68*, 2255-2258.

Witherden, EA, Bajanca-Lavado, MP, Tristram, SG, & Nunes, A (2014) The role of inter-species recombination of the *ftsI* gene on the dissemination of altered

penicillin-binding protein 3 mediated resistance in *Haemophilus influenzae* and *Haemophilus haemolyticus*. *J Antimicrob Chemother, In Press*.

Xie, J, Juliao, PC, Gilsdorf, JR, Ghosh, D, Patel, M, & Marrs, CF (2006) Identification of new genetic regions more prevalent in nontypeable *Haemophilus influenzae* otitis media strains than in throat strains. *J Clin Microbiol, 44*, 4316-4325.

Zapun, A, Contreras-Martel, C, & Vernet, T (2008) Penicillin-binding proteins and beta-lactam resistance. *FEMS Microbiol Rev, 32*, 361-385.

Zerva, L, Biedenbach, DJ, & Jones, RN (1996) Reevaluation of interpretive criteria for *Haemophilus influenzae* by using meropenem (10-microgram), imipenem (10-microgram), and ampicillin (2- and 10-microgram) disks. *J Clin Microbiol, 34*, 1970-1974.